RNA interference (RNAi) enables flexible and dynamic interrogation of entire gene families or essential genes without the need for exogenous proteins, unlike CRISPR-Cas technology. Unfortunately, isolation of plants undergoing potent gene silencing requires laborious design, visual screening, and physical separation for downstream characterization. Here, we developed an adenine phosphoribosyltransferase (APT)-based RNAi technology (APTi) in Physcomitrella patens that improves upon the multiple limitations of current RNAi techniques. APTi exploits the prosurvival output of transiently silencing APT in the presence of 2-fluoroadenine, thereby establishing survival itself as a reporter of RNAi. To maximize the silencing efficacy of gene targets, we created vectors that facilitate insertion of any gene target sequence in tandem with the APT silencing motif. We tested the efficacy of APTi with two gene families, the actin-dependent motor, myosin XI (a,b), and the putative chitin receptor Lyk5 (a,b,c). The APTi approach resulted in a homogenous population of transient P. patens mutants specific for our gene targets with zero surviving background plants within 8 d. The observed mutants directly corresponded to a maximal 93% reduction of myosin XI protein and complete loss of chitin-induced calcium spiking in the Lyk5-RNAi background. The positive selection nature of APTi represents a fundamental improvement in RNAi technology and will contribute to the growing demand for technologies amenable to high-throughput phenotyping.

Loss-of-function studies have long served as building blocks of our understanding of biological processes.

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such as Cas9, and enables reversible reduction of protein levels through targeted degradation of mRNA (Small, 2007). RNAi’s ease of use and flexibility lends itself as an indispensable complement to genetic editing techniques. Traditionally, RNAi in plants is induced through expression of long inverted repeats that self-base pair to form double-stranded RNA (dsRNA), which is then processed into multiple small interfering RNAs (siRNAs) and targeted to complementary sequences within mRNA (Chuang and Meyerowitz, 2000; Hannon, 2002; Baulcombe, 2004). Importantly, a single dsRNA targeting one gene can simultaneously silence multiple genes with sufficient similarity, or a single dsRNA can be generated that contains multiple gene targets in tandem for simultaneous silencing (Vidali et al., 2007; Li et al., 2013). The expression of dsRNAs can be specifically modulated, either through induction or unique promoters, which allows developmental and cell-type-specific reduction of even essential gene products (Byzova et al., 2004; Nakaoka et al., 2012; Miki et al., 2015; Liu and Yoder, 2016). Despite these advantages, RNAi is hindered by variable efficacy of target gene silencing and potential off-target effects (Xu et al., 2006). However, some argue the prevalence of off-target effects is overstated (Zimmer et al., 2019), and importantly an ideal RNAi experiment should demonstrate rescue of the gene silencing phenotype (Vidali et al., 2009, 2010; Ding et al., 2018). Work using artificial microRNAs (amiRNAs) attempts to circumvent the limitations of traditional dsRNA-based RNAi by engineering a single siRNA (Schwab et al., 2006; de la Gutiérrez-Nava et al., 2008). Although amiRNA technology ameliorates possible off-targets derived from the initial dsRNA, evidence in Physcomitrella patens shows generation of additional siRNAs upon cleavage of the amiRNA target, potentially negating the specificity of the amiRNA (Khraiwesh et al., 2008). Furthermore, amiRNAs display variable silencing efficiency, thereby necessitating screening of multiple amiRNAs and limiting experimental throughput (Li et al., 2013; Zhang et al., 2018). To date, no RNAi method addresses another potential source of variability of silencing: transcriptional silencing of the RNAi transgene itself, likely mediated by Dicer-like3 (DCL3; Morel et al., 2000; Fusaro et al., 2006; Small, 2007).

Generating and characterizing loss-of-function mutants using gene silencing methods is fundamentally a two-step process: (1) the implementation of a specific gene-silencing technique; and (2) the identification and isolation of the target(s) undergoing gene silencing for further characterization. Recent advancements in gene-silencing technologies have focused on enhancing the flexibility and robustness of step 1 (Hauser et al., 2013; Zhang et al., 2018) but fail to address the practical limitations imposed by step 2. Identification of actively silencing mutants has been eased by coupling the silencing target of interest to a reporter, such as a nuclear-localized fluorescent protein (Bezannilla et al., 2005; Vidali et al., 2007, 2010; van Gisbergen et al., 2018; Zhang et al., 2018). When paired with an automated or semiautomated image acquisition and analysis pipeline the burden of identifying silencing mutants is substantially mitigated (Wu and Bezanilla, 2012; Galotto et al., 2019). Nevertheless, the tedious of manually isolating silencing plants remains. This limitation is a consequence of traditional gene-silencing construct design, whereby the silencing module is regulated independently from the selectable marker. A typical gene-silencing experiment will contain a heterogeneous population of actively silencing plants, presumably a result of the plant silencing the exogenous silencing module to rescue itself (Fusaro et al., 2006; Khraiwesh et al., 2010). This transcriptional-based silencing not only increases variability of target silencing, but when coupled with visual screening, it exacerbates the manual labor required to isolate mutants. Therefore, downstream characterization of silencing plants, such as reverse transcription quantitative PCR and immunoblots, is stymied. Furthermore, certain reporter-based silencing limits the experimental scope to testing only within established reporter lines (Bezanilla et al., 2003; Nakaoka et al., 2012). For example, using a fluorescent reporter to infer silencing could complicate the subsequent use of other fluorescent outputs, such as biosensors or fluorescent fusions for protein localization, to characterize mutant function.

Here, we generated a modular, Gateway-based RNAi construct that couples silencing any gene(s) of interest in tandem with silencing of adenine phosphoribosyltransferase (APT-interference/APTi). This approach results in near-undetectable levels of background (non-silencing) transformants, thereby trivializing the identification and isolation of actively silencing plants to the simple observation and harvesting of all living plants. Unlike traditional antibiotic positive selection followed by visual screening, our new construct simultaneously selects for transformation and silencing through positive selection alone. We achieved this by exploiting the ubiquitous selectable marker system APT, which converts purine analogs to cytotoxic nucleotides (Schaff, 1994). The APT loss-of-function selection system has been successfully used in plants (Moffatt and Somerville, 1988; Charlot et al., 2014), mammals (Schaff et al., 1990), and bacteria (Levine and Taylor, 1982), but to our knowledge, all experiments with APT involve stable genetic mutants. To quantitatively evaluate the performance and robustness of our APTi system, we separately targeted two gene families, myosin XI (a,b) and Lyk5 (a,b,c), using the plant model organism *P. patens*. Targeting the myosin XI family using APTi resulted in an exclusively mutant surviving population, displaying the characteristic loss-of-growth phenotype (Vidali et al., 2010) and a >90% reduction of target myosin XI protein abundance. Additionally, APTi enabled rapid functional analysis of the previously uncharacterized *P. patens* Lyk5 chitin receptor family, which resulted in complete desensitization of *P. patens* to chitin. Together, APTi-based silencing efficacy far surpasses other dsRNA-based methods (Vidali et al., 2007, 2010; Nakaoka et al., 2012;
Based on endogenous APT activity, Guo et al. (2019), is comparable to silencing efficiencies for optimized amiRNAs (Zhang et al., 2018), and simplifies downstream analysis for functional genomics.

RESULTS

Development of a Positive Selection RNAi Methodology Based on Endogenous APT Activity

Current RNAi methods produce a range of phenotype severity due to variable silencing efficiencies. This variability necessitates optimization experiments that screen fluorescent reporters to maximize silencing by gene sequence targets (Li et al., 2013; Zhang et al., 2018). We sought to simultaneously improve RNAi silencing efficiency and streamline characterization of RNAi mutants by coupling silencing of the gene target with a survival advantage. Previous work in P. patens directly coupled the sequence of a stably integrated, constitutively expressed reporter, such as a fluorescent protein and/or GUS, to the gene target sequence in inverted repeats (Bezanilla et al., 2003, 2005; Nakaoka et al., 2012). Therefore, expression would result in dsRNA formation and coreduction of the intracellular reporter and the coupled target gene. We reasoned that coupling of a lethal reporter sequence in tandem with any other gene sequence would result in maximal cosilencing to ensure silencing of the lethal reporter, thereby promoting survival.

The APT gene has been frequently used as a reporter to evaluate gene-targeting efficiency (Schafer, 2001; Charlot et al., 2014). Functional APT converts adenine analogs, such as 2-fluoroadenine (2-FA), to cytotoxic nucleotides (Schaff, 1994). Therefore, sufficient reduction of APT activity will impart resistance to 2-FA, but this has only been demonstrated in genetic knockouts. To test if silencing of PpAPT (Pp3c8_16590) effectively conferred survival to plants grown on 2-FA, we inserted an APT-targeting sequence into a previously developed RNAi vector that also contains a reporter targeting sequence (Bezanilla et al., 2005). To maximize silencing efficacy, we generated an APT targeting sequence consisting of the 5′ untranslated region (UTR; 179 bp) and first 210 bp of the APT gene. The APT silencing construct (APT-RNAi) conferred resistance to wild-type P. patens cultured on 1.25 μg mL⁻¹ 2-FA, whereas no plants survived on 2-FA when transformed with a control plasmid lacking the APT silencing sequence (control-RNAi; Fig. 1A). This result clearly establishes survival on 2-FA paired with APT targeting as a conspicuous phenotypic reporter of active silencing.

Our previous APT silencing experiment demonstrated feasibility, but further development of the technique was constrained by the available construct. As mentioned above, the first iteration of APTi was inserted into a vector created specifically for RNAi (Bezanilla et al., 2005). This construct included a pair of inverted Gateway sites coupled to a target sequence (GUS) for an internal reporter of active silencing. The reporter sequence targeted a nuclear-localized GFP:GUS fusion protein, thereby requiring the use of a special transgenic line for RNAi experiments (Bezanilla et al., 2003). In principle, our APTi would not require any specific moss line and instead would permit the researcher to perform RNAi experiments in any genetic background. Therefore, we replaced the GUS reporter target sequence with the APT target sequence while maintaining the inverted Gateway cassettes and loop region and named the construct pGAPi (Fig. 1B). This construct allows straightforward insertion of any gene sequence and ensures fusion to the APT target, thus permitting inference of gene silencing within surviving plants. Importantly, entire gene families can be targeted by simple insertion of a conserved sequence of ~400 bp or concatenation of individual target sequences followed by cloning into our pGAPi (Fig. 1B). Additionally, we created another APTi construct, named pAPI, which lacks the internal Gateway cassettes to serve as a “positive control” for any RNAi experiment (Fig. 1B). Together, these constructs serve as the foundation for the APTi system and advance positive selection as an effective reporter of gene silencing.

APTi Experimental Design for High-Throughput Phenotyping

Although our APTi system clearly selects for silencing plants cultured over a 2-week period by visual inspection (Fig. 1A), we sought to establish a rapid, semiautomated microscopy assay using APTi for plant phenotyping. A fundamental attribute of any high-throughput assay is the effective and automated discrimination between objects of interest and background that co-occupy the same space. Our APTi system simplifies the problem of automated separation. Unlike fluorescence reporter systems where the reporter signal is continuous and exhibits natural variation, APTi reduces separation of silencing and nonsilencing plants to the binary decision of alive or dead. We reasoned that chlorophyll autofluorescence could function as a proxy for plant survival, thereby enabling automated detection of alive, and therefore actively silencing, plants. In principle, all plants successfully transformed with a construct silencing APT, pAPI (Fig. 1B), will survive on 2-FA medium, whereas plants transformed with an RNAi construct silencing a nonexistent reporter, pUGi (Bezanilla et al., 2005), will die.

We tested this by using the experimental design illustrated in Figure 2. P. patens protoplasts were transformed with either pAPI or pUGi, allowed to regenerate for 4 d, then transferred to growth medium supplemented with 1.25 μg mL⁻¹ 2-FA. Importantly, during the optimization phase of this assay, we observed substantial variability in the outcome of 2-FA selection. We empirically determined that starting the selection 4 d posttransformation and making the 2-FA selection plates fresh on the day of selection mitigated
essentially all experimental variability. We strongly suggest first optimizing the 2-FA selection concentration when applying the APTi system, as our results were all obtained using a single lot of 2-FA from Oakwood Chemical.

Following 4 d on 2-FA-supplemented growth medium, cultures were removed from the growth chamber for protein extraction and microscopy analysis (Fig. 2B). To facilitate high-throughput phenotyping and remove human bias, we used an epifluorescent microscope equipped with an automated stage integrated with image tiling and stitching software. This enabled large region-of-interest (ROI) acquisition, with the size of the ROI only constrained by the memory available to the computer. For our experiments, every composite image was constructed from a 12 × 12 grid of single images, with a 15% overlap, which corresponds to an ROI surface area of approximately 1.8 cm².

To simplify downstream image segmentation, all plants were stained with calcofluor to label the cell wall before imaging. Each individual image contained two channels, the chlorophyll autofluorescence and the calcofluor signal. Visualization of the chlorophyll signals of APT-RNAi and control-RNAi clearly reveals the efficacy of the APTi system: not a single control-RNAi plant survives and does not markedly grow beyond the initial regeneration size (Fig. 2A). This result is reproducible, as the chlorophyll signal across three independent experiments for control-RNAi plants consistently clustered below a characteristic intensity (Fig. 2A). We used chlorophyll intensity parameter as a threshold, from which we functionally partitioned a mixture of plants on a plate into alive (silencing) and dead (nonsilencing) plants (Fig. 2A). The alive or dead classification ensured that the morphometric parameters extracted from the image analysis pipeline were confined to only alive, and therefore silencing, plants. Therefore, it stands to reason that when using our APTi system any statistically supported observed difference between control and treatment plants is directly attributable to silencing of the targeted gene(s).

Figure 1. Proof of principle and construction of APT-based RNAi (APTi) vectors to enable positive selection of actively silencing plants. A, Illustration of the APT-interference positive selection principle: plants are transformed with a vector that creates a long dsRNA hairpin targeting the APT gene, thereby reducing endogenous APT levels and subsequent production of cytotoxic nucleotides when supplemented with 2-FA. Targeting APT using RNAi is sufficient for transformed plants to grow on standard PpNH₄ medium supplemented with 1.25 μg mL⁻¹ 2-FA. B, Schematics of the APT-based RNAi vectors. The pAPI (plasmid APT RNAi) and pGAPI (plasmid Gateway APT RNAi) constructs were created using the pUGi and pUGGi vectors, respectively, from Bezanilla et al. (2005) as templates. The thin black arrows indicate the direction of the open reading frame, and the inverted repeat regions of both constructs are flanked by a constitutive maize (Zea mays) ubiquitin promoter (thick black arrow) and a NOS terminator sequence (black rectangle). Both constructs target the 5’UTR (blue rectangle, 179 bp) and CDS (green rectangle, 210 bp) of the APT gene. pAPI contains only the loop (red rectangle, 402 bp in pAPI, 392 bp in pGAPI) region within the inverted repeat, whereas pGAPI contains inverted Gateway sites to facilitate insertion of target sequence. The target may be unique to gene X, or if the target sequence is conserved, the user can simultaneously silence multiple targets in tandem with APT silencing, thereby enabling survival of silencing plants.
The APTi System Silences the Myosin XI Family with High Efficacy

We demonstrated that silencing of APT permits potent positive selection of actively silencing plants (Fig. 2A), but we sought to extend APTi to mutant analysis. We reasoned that insertion of a target sequence into our APTi vector (Fig. 1B) would produce tandem silencing of APT and the target gene. To test this hypothesis, we exploited the well-characterized transient myosin XI(a\textsubscript{1}b) RNAi mutant that produces a dramatic loss of polarized growth phenotype (Vidali et al., 2010). Furthermore, the myosin XI(a\textsubscript{1}b) mutant was previously generated using a fluorescent reporter-based RNAi strategy, allowing a direct comparison of methodology.

In P. patens, the myosin XI gene family contains two functionally redundant isoforms, XIa and XIb, which are both expressed in protonemata (Vidali et al., 2010). To simultaneously silence both myosin XI genes and allow for rescue experiments, we created an APTi construct that contains a concatenated 5′ UTR sequence derived from both isoforms of myosin XI. We named this construct “myoUTi(a+b),” and it resulted in a striking recapitulation of the myosin XI phenotype (Fig. 3A). Impressively, nearly every surviving plant manifested the characteristic “bunch of grapes” morphological phenotype (Supplemental Fig. S1). This is exemplified by the relatively narrow distributions of the APTi myosin XI knockdown in the two morphology parameters, solidity and area (Fig. 3B). We speculate the survival advantage inherent to our APTi method could reduce phenotypic variability sometimes observed in RNAi experiments.

We inquired whether this phenotype could be rescued in the APTi system by cotransforming the myoUTi(a+b) construct with a plasmid expressing only the coding sequence of myosin XIa, “XIa CDS,” thereby evading the silencing construct that targets the 5′ UTR. We observed near-complete rescue of the myosin XI phenotype, demonstrating an absence of off-target effects and, more importantly, highlighting the rapid phenotyping utility of the APTi system when coupled with P. patens. Within 8 d, our APTi system isolated through positive selection a relatively homogenous population of mutant plants, which were amenable to rescue. Our APTi system produced average myosin XI mutant morphological parameters similar to a myosin XI mutant derived from RNAi using an internal GFP reporter system (Vidali et al., 2010) but without nonsilencing background plants. Together, these results support our prediction that aberrant morphologies
observed using the APTi system are caused by silencing of the conjugated target, in our case myosin XI. As our APTi approach removes essentially all background, we asked if the previously intractable problem of reliable protein quantification could be trivialized to harvesting all material present on the 2-FA plate. To accurately quantify myosin XI protein abundance using our APTi method, we first established the linear range of our antibodies. To reflect our RNAi experimental conditions, we transformed wild-type moss with pAPi, grew it on medium supplemented with 2-FA, then harvested the entire plate at 8 d posttransformation. Total protein was determined using a Bradford microplate microassay (Bio-Rad), then a range of total protein (1–20 μg) was probed using both an in-house developed antibody against myosin XIa’s coiled-coil tail (CCT) region and a publicly available anti-α-tubulin antibody (DSHB: AA4.3). This approach revealed an approximate linear range for both antibodies from 5 to 20 μg total protein (Supplemental Fig. S2A). Importantly, under equivalent conditions, our limit of detection for endogenous, wild-type myosin XI was approximately 1 μg total protein (Supplemental Fig. S2A). This step is essential, as it allows for confident, semiquantitative estimation of the extent of protein reduction. Without this, the wild-type protein of interest could be loaded at or very near the limit of detection, resulting in a complete absence of protein signal in the RNAi condition, even if the true reduction is modest. We performed an APTi experiment for myosin XI, as shown in Figure 3, and harvested at 8 d posttransformation. Every condition was implemented in at least duplicate to allow for both harvesting of plants and our phenotyping assay (Fig. 2). In this way, the results of our protein analysis directly reflected the internal protein abundance and corresponding morphologies we observed in the phenotyping assay (Fig. 3A). We observed a dramatic decrease of myosin XI family in P. patens. A, Representative images of 8-d-old plants regenerated from protoplasts transformed with APTi vectors targeting APT alone (pAPi), APT in tandem with a concatenated 5′ UTR sequence for myosin XIa and XIb (myoUTi), targeting a nonexistent GUS sequence (pUGi), or myoUTi cotransformed with a construct overexpressing myosin XIa’s coding sequence. All images are cropped from composite images that capture a large sample area for an individual condition, as shown in Figure 2A. For every condition, each image corresponds to an independent experiment. Chlorophyll autofluorescence is colored magenta, and calcofluor signal is colored green. Scale bar = 100 μm. B, Quantification of morphometric parameters solidity and area from three independent experiments. Area is normalized to the mean area of the pAPi condition, which represents near wild-type growth morphology. pAPi, n = 270; myoUTi, n = 125; myoUTi + XIa CDS, n = 178. Lowercase letters indicate statistical difference (P < 0.001) between groups as determined by one-way ANOVA with post-hoc Tukey test. C, Immunoblots demonstrating the reduction and restoration of myosin XI protein levels when using the APTi system. Each experiment represents an independent transformation and subsequent plant harvesting and immunoblotting. Ten micrograms of total protein was loaded per lane. Myosin XI was probed using a polyclonal antibody generated against the myosin XIa CCT fragment from P. patens, and α-tubulin was used as a loading control. D, Densitometry of immunoblot signals was performed using ImageJ and shows an ~90% reduction of myosin XI and a complete rescue of myosin XI protein levels when compared to the pAPi control.
XI in the “myoUTi” condition, which when normalized to α-tubulin results in a maximal 93% reduction relative to the control (“pAPi”). Additionally, the rescued mutant morphology precisely corresponds to an almost wild-type restoration of myosin XI levels (Fig. 3, C and D). Of note, these results were consistent across independent experiments and resulted in an average silencing efficiency of 90% (Fig. 3, C and D). We explored the longevity target silencing using APTi by probing myosin XI at 2 weeks posttransformation. Although less potent than at 8 d posttransformation (Fig. 3), myosin XI levels were still substantially reduced (Supplemental Fig. S2, B and C), opening the possibility for long-term phenotyping. At the longer time, the rescue condition was noticeably weaker than at the short time point. We attribute this to loss of the myosin XI expression plasmid, as it is not under selection. Taken together, these data establish that aberrant phenotypes observed using our APTi system are directly attributable to reduction of target protein abundance.

**APT-Based Silencing of the Lyk5 Family Eliminates P. patens Perception to Chitin Oligosaccharides**

Despite the success of the APTi system when applied to myosin XI, we could not discount the possibility that myosin XI was particularly amenable to APTi and alternative gene targets would yield less favorable results. To address this concern, we reasoned that the Lyk5 gene family of *P. patens* presents an excellent candidate to test the robustness and potential for functional discovery using APTi. In Arabidopsis (*Arabidopsis thaliana*), LYK5 is a member of a gene family of lysin-motif-containing proteins and functions as a receptor for the fungal polysaccharide chitin, likely in concert with chitin elicitor receptor kinase1 (CERK1; Cao et al., 2014). LYK5, together with LYK4 in Arabidopsis, functions redundantly in chitin perception, with the lyk4 lyk5 double mutant losing all chitin sensitivity (Cao et al., 2014). Like Arabidopsis, application of chitin to *P. patens* elicits calcium transients (Galotto et al., 2020), suggesting APT-RNAi alone has no influence on the output of our assay. When treated with Lyk5-RNAi, plants were rendered entirely insensitive to chitin (Fig. 4B). Furthermore, the characteristic dose dependence of calcium signal on external chitin was abolished (Fig. 4C), and calcium oscillations were nonexistent when targeting the entire Lyk5 family (Fig. 4D). Our preliminary experiments silencing only two of the three Lyk5 genes showed calcium spikes in response to chitin, suggesting we are targeting all three genes with our Lyk5-RNAi construct. Additionally, as only the double knockout lyk4 lyk5 mutant in Arabidopsis loses all chitin sensitivity (Cao et al., 2014), we conclude our APTi-based silencing of the Lyk5 family is functioning with high efficiency to phenocopy a double knockout mutant in Arabidopsis. Importantly, these results elucidate functional homology of the LYK4/5 genes between Arabidopsis and *P. patens*, whereby Lyk5 functions at the level of chitin perception. Previous work has demonstrated that *P. patens* CERK1 rescues the Arabidopsis cerk1 mutant (Bressendorff et al., 2016), strongly supporting a conserved mechanism of chitin sensing. All together, our findings demonstrate the robustness and versatility of our APTi system for mutant isolation and characterization.

**DISCUSSION**

RNAi offers an invaluable complement to traditional gene knockout studies. However, substantive advancements in RNAi methods are trailing the explosion of CRISPR/Cas-based technologies. Here, we established the first survival-based RNAi methodology that robustly selects for actively silencing plants. We accomplished this by engineering vectors that elicit a prosurvival response when processed by the organism’s endogenous RNAi machinery. Using the previously characterized myosin XI mutant in *P. patens* as an
initial case study, we showed that in tandem fusion of a myosin XI target with the prosurvival sequence resulted in potent selection of morphologically mutant plants. We demonstrated that surviving plants actively targeting myosin XI through our novel vectors contained approximately 7% of normal myosin XI protein abundance. Additionally, using APTi facilitated the silencing of the Lyk5 family and resulted in moss plants completely insensitive to chitin-induced calcium spikes. To our knowledge, this is the first evidence demonstrating the function of Lyk5 in P. patens and supports the notion of conservation in fungal perception between vascular and nonvascular plants. Our APTi technology represents a dramatic improvement in silencing efficacy and experimental implementation over previous RNAi methods.

RNAi has been extensively employed in the model moss P. patens for both discovery and validation of gene function (Vidali et al., 2007, 2010; Augustine et al., 2008, 2011; Prigge et al., 2010; Wu et al., 2011; Miki et al., 2015; Bascom et al., 2019). We attribute the popularity of dsRNA-based RNAi in P. patens to a method that uses an internal fluorescent reporter of RNAi to obtain results in 1 week (Bezanilla et al., 2003). We sought to fundamentally improve upon RNAi reporters by creating a reporter where survival itself is indicative of active silencing. We demonstrated the feasibility and effectiveness of this approach by silencing the APT gene in the presence of 2-FA in the medium. Furthermore, we engineered two plasmids that facilitate insertion of any target of interest in tandem with the APT-silencing sequence. We call this approach APT-based RNAi, or APTi. To achieve rapid phenotyping using APTi, all experiments were performed transiently. Modifications of the APTi plasmids to promote stable integration and induction of the silencing cassette represent an important area of future work. Both APTi plasmids and the myosin

Figure 4. Simultaneous silencing of the Lyk5 gene family with APTi abolishes chitin-induced calcium transients. A, Schematic of APTi-based functional assay of Lyk5. Control RNAi plants treated with chitin elicit calcium transients, visualized with GCaMP. Simultaneous depletion of the Lyk5 receptor family (a, b, c) using APTi desensitizes the plants to chitin, resulting in loss of calcium spikes. B, Eight-day-old plants treated with 20 mg mL⁻¹ chitin oligosaccharides. Time stamps are mins. Dark blue indicates plant autofluorescence, where aqua to red reflects an increase of calcium signal. Wild-type represents GCaMP6 in the Gransden background. C, Dose response of P. patens' calcium signal to chitin application for control RNAi plants and plants transformed with an APTi construct targeting all three Lyk5 genes. Time-averaged mean gray value (left plot) and the SD of the time-averaged mean gray value (right plot) are normalized by \( (F_0 - F)/F_0 \) for both conditions. All data points are the mean of at least three plants pooled from three independent experiments. D, Characterization of calcium transients within a 30-min observation period, as in A, across multiple concentrations of chitin. All points represent an individual plant, pooled across three independent experiments. Bars represent the mean ± the SEM. Each condition was compared against itself across concentrations, with an asterisk representing a statistical difference between that condition and its corresponding water control. Statistics were performed with a Kruskal-Wallis one-way nonparametric ANOVA, followed by a Dunn’s multiple comparisons test and indicated by asterisks (*\( P < 0.001 \)).
XI-RNAi plasmid are publicly available from the plasmid repository Addgene.

We obtained potent positive selection of actively silencing plants in *P. patens* by engineering RNAi vectors that exploit the function of the APT gene. However, the physiological consequences of APT silencing remain in question. Although it is a salvage enzyme, APT’s function presents a more energetically efficient means of nucleotide production than de novo synthesis (Ashihara et al., 2018). Consequently, knockouts of APT in vascular plants demonstrate severe defects in pollen germination and pollen tube growth, presumably a result of impairing the energy-intensive fast growth of the pollen tube (Moffatt and Somerville, 1988; Zhou et al., 2006). Interestingly, an alternative mutant allele of APT that results in partial reduction of APT activity imparts enhanced growth and stress tolerance (Sukrong et al., 2012). We suspect this hypomorphic allele better represents the internal state of our APT-silenced plants. Taken together with our observations of APT-silencing plants, reproducing results achieved with an orthogonal RNAi system and APT-RNAi plants displaying calcium spikes consistent with wild-type, we conclude the reduction of APT results in no clear physiological defects within the scope of our assays.

We expect our APTi strategy to be applicable to other organisms given the ubiquity of the APT gene (Schaff, 1994). Like other organisms, such as in humans, *P. patens* contains only one copy of APT, making it especially amenable to the APTi strategy as we showed. Interestingly, the vascular plant Arabidopsis contains five APT genes (Allen et al., 2002). We suspect APTi could be applied in Arabidopsis by constructing an APT-silencing module comprised of concatenated sequences targeting specific isoforms. Additional work is necessary to translate to other systems, but we submit that our efforts establishing APTi in *P. patens* will greatly benefit the community in understanding fundamental and conserved biological processes (Orr et al., 2020).

Previous work using the fluorescent reporter-based RNAi has identified silencing plants based on loss of fluorescence (Bezanilla et al., 2003). However, slight reduction in fluorescence confounded interpretation because it could be attributed to natural variation in the reporter signal or reflect a real, but modest, silencing effect. Furthermore, we observed spontaneous loss of reporter signal in the moss reporter line over long periods of continuous propagation. Without careful observation and subcloning to remove chimeric reporter cultures, a researcher could inadvertently conclude silencing when none is occurring. Our APTi system simultaneously removes the requirement of a dedicated moss reporter line and dismisses any ambiguity inherent to a continuous reporter signal. We demonstrated the utility of this advancement in our investigation of Lyk5 by transforming our APTi-based construct into a moss line expressing the calcium sensor GCaMP6f (Galotto et al., 2020). This saved considerable time, as the previous RNAi methodology would require creation of a new transgenic line that contains the RNAi GFP reporter and a non-GFP-based calcium sensor.

Interestingly, prior RNAi methods can result in background plants that survive antibiotic selection for the plasmid containing the RNAi transgene but do not silence the reporter. This is likely a result of transcriptional gene silencing, whereby the plant rescues itself from RNAi transgene expression, but expression of the independently regulated antibiotic resistance is unmodified (Morel et al., 2000; Fusaro et al., 2006; Small, 2007). We hypothesize our method enhances silencing efficiency by engineering a fitness punishment for the organism to silence the RNAi transgene. To this end, with APTi survival on 2-FA is directly coupled to expression of the APT-RNAi transgene. Therefore, the organism cannot survive if it silences the expression of the RNAi cassette, thus ensuring expression of the APT+ gene target hairpin. Although we did not determine the extent of APT silencing, we know the reduction is sufficient to promote survival on 2-FA and produce a 90% reduction of a fused target, myosin XI, or complete loss of detectable calcium signal when all three Lyk5 genes are targeted.

We speculated that the enhanced fitness benefit imposed by our APTi system will result in more consistent and potent silencing efficiencies of target genes. Consistent with this, we observed a homogenous population of mutants evidenced by a 90% reduction of endogenous myosin XI and total loss of chitin sensitivity when targeting myosin XI(a,b) and Lyk5(a,b,c), respectively. APTi offers a noticeably higher silencing efficacy when compared to previous reports using dsRNA and GFP-based reporters in *P. patens* (Vidal et al., 2007, 2010; Nakaoka et al., 2012). Importantly, previous reports could only estimate protein silencing based on a small subset of individual plants deliberately chosen by the experimenter, which may not accurately reflect the average silencing effect (Vidal et al., 2007, 2009, 2010; Augustine et al., 2008). This microscope-based methodology was required because mutants resulting in small morphologies failed to yield adequate plant material for immunoblots and were surrounded by nonsilencing background plants. We demonstrated that APTi’s positive selection enables simple harvesting of the entire plate that can be easily scaled for reproducible protein quantitation. Based on our observed myosin XI knockdown, our APTi silencing efficacy is comparable to the most effective amiRNAs (Zhang et al., 2018) but without the need for prior engineering and screening of multiple amiRNAs. Furthermore, performing our Lyk5 functional assay with prior GFP-based RNAi would require an additional time-consuming and tedious pre-screening step, where actively silencing plants are physically isolated from the nonsilencing background. With APTi, any surviving plant can be chosen at random for functional analysis, greatly enhancing the throughput and likely the consistency of the results.

We showed that the APTi system is well suited for high-throughput phenotyping. We fully anticipate this...
area to be iteratively improved, not just with respect to the volume of acquisition but with increased computational sophistication. The large obtainable data sets are ripe for both classic exploratory data methods and cutting-edge deep-learning techniques. For example, we analyzed living plants by first segmenting the images by traditional thresholding. We then filtered and classified the hundreds of plants based on a characteristic biological feature, chlorophyll autofluorescence, which we derived from the control-RNAi dying population. Although less intuitive, deep learning offers the possibility of automating image segmentation and classification, perhaps resulting in discovery of novel mutant features (Moen et al., 2019).

CONCLUSION
This work represents a fundamental transition from visual screening for RNAi plants to positive selection of actively silencing plants. We achieved this by engineering vectors that produce a single hairpin RNA targeting the APT gene and any other genes of interest in tandem. This results in effective isolation of all surviving plants undergoing RNAi of the target gene when grown in the presence of 2-FA. Importantly, the efficacy of gene silencing was consistently greater with the APTi system, maximal 93% reduction of target protein, than previous reports silencing the same myosin XI genes using a fluorescent screening method. Additionally, with APTi, we simultaneously silenced the Lyk3 gene family (a,b,c) and demonstrated its requirement for perception of chitin oligosaccharides in P. patens. We believe our APTi system provides a flexible, fast, and effective platform with unprecedented low background and variability to facilitate high-throughput characterization for loss-of-function mutants.

MATERIALS AND METHODS

Plant Materials and Culture Conditions
Three Physcomitrella patens lines were used in this study: (1) NLS4 (Bezanilla et al., 2003) in Figure 1A; (2) CCAMPF6, called wild-type in Figure 4 (Galotto et al., 2020); and (3) wild-type Gransden (Ashton et al., 1979) in all other experiments. All lines were cultured as previously described (Vidal et al., 2007). In brief, tissue was propagated weekly by homogenization and transferred to solid PpNH4 medium overlaid with cellophane. Cultures were grown at 25°C under long-day light (90 μmol m−2 s−1) conditions (16 h light, 8 h dark).

APT-Based RNAi Construct Design
The APT transcript fragment (Phytozone: Pp3c8_16590) containing the 5’ UTR and CDS was amplified by PCR with forward (APTi_BSK_F) and reverse (APTi_BSK_R) primers and cloned into pBlueScript K+ using restriction enzymes SacI/EcoRV (generating the APT pBSK+ plasmid). The pUGGii Gateway cassette and loop domain lacking the GUS regions was amplified in two pieces using PCR with forward (APT_BSK_F) and reverse (APT_BSK_R) primers (both reactions at 60°C, 2 min elongation), and both fragments were ligated individually into pBlueScript K+ (generating the Gateway F pBSK+ and Gateway R pBSK+ plasmids). The pUGGii loop was amplified by PCR with primers Loop_F/R and inserted into pBlueScript K+ (generating the Loop pBSK+ plasmid). The APT and Loop pBSK+ constructs were transformed into DH5a Escherichia coli, whereas both Gateway pBSK constructs were transformed into cdbB Echerichia coli. All constructs were blue-screened for successful clones by plating transformants on LB + Car + Chlor plates and adding 40 μL each of isopropylthio-β-galactoside and X-Gal. Next, using the SacI/Xhol restriction sites, the Gateway F fragment was inserted into the Gateway R pBSK+ backbone, generating the complete Gateway pBSK+ plasmid. Importantly, this construct contains the entire loop region derived from pUGGii, as the two intermediate Gateway constructs discussed above each contained half of the loop region. It was necessary to isolate the pUGGii loop in its own plasmid to create an additional control plasmid lacking the Gateway cassettes.

The first APT fragment was then excised from APT pBSK+ using HindIII/SwaI, then transferred into the Gateway pBSK+ plasmid, and cut with HindIII/Pmel (generating Gateway-1APT pBSK+). Next, the second APT fragment was excised from APT pBSK+ and transferred into the Gateway-1APT pBSK+ plasmid using SacI/SwaI sites (generating Gateway-2APT pBSK+). The Gateway-2APT fragment was digested and then ligated into the pUGGii backbone using SacI/KpnI sites, generating the pAPi plasmid. To generate our positive control plasmid without Gateway sites, two APT fragments were inserted into the Loop pBSK+ plasmid using the same procedure as described above—first by HindIII/SwaI and HindIII/Pmel sites, then by SacI/SwaI sites—producing the Loop-2AP Ti pBSK+ plasmid. Then the Loop-2APT fragment was cloned into the pUGGii backbone using SacI/KpnI sites, generating the pAPi plasmid. At all points in this process where new plasmids were made by restriction-based cloning, those plasmids were confirmed by restriction and sequence analysis.

The myoUTi:pGAPi construct was created by extracting the previously published, concatenated 5’ UTR targeting sequences (Vidal et al., 2010) from its RNAi destination vector via Gateway BP reaction, then inserted into our new pGAPi construct via a Gateway LR reaction. The transient myosin XI-RNAi phenotype was rescued by expressing the myosin XIa coding sequence (Vidal et al., 2010). All three APTi plasmids are publicly available from Addgene (pGAPi, #127547; pAPi, #127548; myoUTi:pGAPi, #127549), and sequences are also available at GenBank (pGAPi, MK975290; pAPi, MK975291; myoUTi:pGAPi, MK975292).

APTi Phenotyping Assay
One-week-old moss was protoplasted and transformed as described (Liu and Vidal, 2011), with transformed protoplasts being suspended in liquid plating medium and plated at 1.4 × 105 protoplasts per 100-mm petri dish of protoplast regeneration medium for the bottom layer. Each condition was plated at least duplicate, with the myoUTi condition being plated in triplicate to allow enough plant material of the mutant plants to be harvested for immunoblotting and imaging. Four days posttransformation, the cellophane of each plate were transferred to growth medium (PpNH4) supplemented with 1.25 μg mL−1 2-FA from a 5 mg mL−1 diethyl sulfoxide stock (Oakwood Chemical). As mentioned previously, each lab should first optimize the 2-FA selection concentration, as our results were all obtained using a single lot of 2-FA from Oakwood Chemical.

Eight days posttransformation plants were stained with 10 μg mL−1 calcofluor from a 1 mg mL−1 water stock (Fluorescent Brightener 28, Sigma) and imaged with a 10X A-Plan (0.25 NA) objective of an epifluorescence microscope (Zeiss Axiovert 200M) coupled to a CCD camera (Zeiss AxioCam MRm). This microscope was equipped with an automated stage and integrated with the AxioVision software through the MosaicX module, enabling precise acquisition, tiling, and stitching of individual images to create a large composite. Our composite images contained 12 × 12 individual images, acquired with a 15% overlap. Each individual image consisted of two channels, the calcofluor and chlorophyll signals. The chlorophyll channel was acquired with a 480/40 bandpass excitation, a 505 long-pass dichroic mirror, and a 510 long-pass emission filter cube and with a fixed 150-ms exposure for all experiments. The calcofluor signal was acquired with a standard DAPI filter and automatically adjusted for each experiment to maximize contrast. Stitched images were first processed and segmented using a custom ImageJ macro (available upon request). Our macro only discarded segmented objects approximately the size of two adjoining protoplasts or smaller, as to not bias against the discovery of small mutants and remove nonsurviving protoplasts. Each segmented image was visually inspected for overplating or truncated plants, and if present they were discarded from further analysis. Subsequent filtering of alive plants and visualization of the data were performed using MATLAB (MathWorks). Statistical testing (one way ANOVA-Tukey) was performed with GraphPad Prism.
Analysis of Myosin XI Protein Abundance

Eight days posttransformation, all tissue was harvested by scraping, flash-frozen in liquid nitrogen, and stored at −80°C. To create protein extracts, the frozen tissue was ground to a powder in liquid nitrogen, then resuspended in extraction buffer (250 mM Suc, 20 mM EDTA, 50 mM PIPES, 150 mM NaCl, 60 mM MgCl2, and 1% [w/v] casein) supplemented with fresh dithiothreitol (2 mM final) and protease inhibitors. The powder from the pAPi condition was resuspended in 300 μL, whereas the myoUTi and rescue conditions were always resuspended in 200 μL extraction buffer. Extracts were vortexed for 15 s, then placed on ice for 30 s, with this repeated twice more. Extracts were then spun at 13,000 rpm for 10 min at 4°C, followed by removal of 175 μL of the clarified supernatant; 120 μL of extract was immediately combined with SDS loading buffer and boiled, then stored at −80°C. The remaining extract was used for total protein determination using the Bradford microplate microassay procedure (Bio-Rad).

To probe myosin XI protein directly, an antibody was developed against a 6× His fusion of the P. patens myosin Xla-CCT (Capralogics). An antibody against α-tubulin was used as a loading control (DSHB: AA4:AA3). The approximate linear range of both antibodies was determined by first loading 1 to 20 μg of total protein from a pAPi-treated moss extract on a 4 to 12% (w/v) Bis-Tris SDS-PAGE gel (Thermo Fisher). Protein was then transferred to nitrocellulose overnight at 4°C, followed by blocking with 5% (w/v) milk in Tris-buffered saline, 0.1% (v/v) Tween 20 at room temperature for 1 h. The nitrocellulose was cut at the 80-kD marker, with the higher M, piece incubated with myosin Xla-CCT primary antibody (1:10,000) and the α-tubulin (0.5 μg mL−1 final) incubated with the lower M, fragment for 1 h at room temperature. Following primary antibody incubation, blots were washed three times in Tris-buffered saline, 0.1% (v/v) Tween 20, incubated in secondary antibody (goat anti-rabbit for Xla-CCT, goat anti-mouse for α-tubulin) at 1:10,000 dilution for 1 h at room temperature, then washed a final three times. Blots were developed using homemade enhanced chemiluminescence reagent, and chemiluminescent images were acquired using an Azure 600 (Azure Biosystems). Densitometry was performed using ImageJ (Schneider et al., 2012) to allow comparison of relative protein abundance.

Lyk5-RNAi Construct Design and Functional Assay

To disrupt the expression of Lyk5 genes in P. patens, an RNAi plasmid was constructed. The first 414 bp of Lyk5a and 418 bp of Lyk5b were amplified using specific primers (Supplemental Table S1) with the Pfusion polymerase (Thermo Fisher), both fragments were joined by overlapping PCR, and the 0.8-kb fragment was gel purified (NucleoSpin gel and PCR clean-up, Macherey-Nagel) and inserted into the entry vector pDONR207 via a BP reaction (Thermo Fisher). To insert the remaining Lyk5 gene, primers were designed for Lyk5c (Supplemental Table S1) that amplified a fragment of exon 1. The corresponding 0.4-kb amplicon was gel purified (Zymogen gel clean DNA recovery kit) and inserted into pDONR207-Lyk5a,b via BamHI. Successful pENT-Lyk5a,b, clones were identified through restriction analysis and sequencing. The concatenated Lyk5a,b,c target sequence was inserted into pGAPI via an LR reaction (Thermo Fisher) between pGAPI and pENT-Lyk5a,b,c, yielding Lyk5-RNAI. The Lyk5-RNAI construct was sequenced verify. Transparent transformation and APT selection were performed as described above.

Calcium imaging and analysis were performed as previously described (Galotto et al., 2020). In brief, plants were mounted on an agar pad containing serial dilutions of chitin oligosaccharides, starting at 20 mg mL−1 (Tokyo Chemical Industry), or water, and imaged with a Zeiss Axio Observer.A1 microscope using a 10× objective lens (NA 0.25). The internal calcium sensor was excited with a mercury lamp X-Cite series 120 PC EXFO, and individual plant images were acquired every 30 s for 30 min within 10 min of initial chitin exposure. Analysis of the calcium time series data was performed using ImageJ and RStudio. Microscope drift was corrected in ImageJ using the ‘StackReg’ plugin (Thévenaz et al., 1998). Next, the area around the plant was selected and the background was cropped. Then a macro applied, for each frame in the series, a thresholding function that equalized the images and masked the area around the plant; the masked images were used to analyze the Ca2+ peaks in chitin-treated plants. In addition, the ImageJ macro recorded the mean gray value of each image. The mean gray values for a time series were averaged (calcium levels) and the S.D of the average calculated (calcium fluctuations). The dose response was calculated in Graphpad Prism by plotting the time-averaged values and S.D for each concentration of chitin oligosaccharides.

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