Knockdown of Genes Involved in Transcription and Splicing Reveals Novel RNAi Targets for Pest Control

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RNA interference (RNAi) is a promising next generation technology for the development of species-specific pest management. The key to successful RNAi based-plant protection is dependent in part on data-driven target gene selection, a challenging task due to the absence of laboratory strains and the seasonality of most pest species. In this study, we aimed to identify novel target genes by performing a knowledge-based approach in order to expand the spectrum of known potent RNAi targets. Recently, the protein-coding genes ncm, Rop, RPII-140, and dre4 have been identified as sensitive RNAi targets for pest control. Based on these potent RNAi targets, we constructed an interaction network and analyzed a selection of 30 genes in the model beetle Tribolium castaneum via injection of dsRNA synthesized by in vitro transcription. Nineteen of these targets induced significant mortality of over 70%, including six that caused 100% lethality. Orthologs of active T. castaneum RNAi targets were verified in the economically important coleopteran pests Diabrotica virgifera virgifera and Brassicogethes aeneus. Knockdown of D. v. virgifera genes coding for transcription factor Spt5, Spt6, and RNA polymerase II subunit RPII-33 caused over 90% mortality in larval feeding assays. Injection of dsRNA constructs targeting RPII-215 or the pre-mRNA-processing factor Prp19 into adult B. aeneus resulted in high lethality rates of 93 and 87%, respectively. In summary, the demonstrated knowledge-based approaches increased the probability of identifying novel lethal RNAi target genes from 2% (whole genome) to 36% (transcription- and splicing-related genes). In addition, performing RNAi pre-screening in a model insect increased also the probability of the identification essential genes in the difficult-to-work-with pest species D. v. virgifera and B. aeneus.

Keywords: Tribolium castaneum, insect pest control, RNAi target prediction, WCR, Diabrotica v virgifera, Brassicogethes aeneus, pollen beetle, biopesticide

INTRODUCTION

The world population is expected to reach ~10 billion people in 2050, necessitating a substantial crop yield increase to meet the global food demand (Johnson and Jones, 2017; Rohr et al., 2019). An area where significant productivity gains can be made is the reduction of crop losses associated with insect pests, which is estimated at ca. 15% at a multicrop and worldwide scale (Oerke, 2006; Fletcher et al., 2020). The primary solution for insect control, the use of chemical pesticides, is facing
challenges like resistance development and growing concerns of undesirable effects on the environment or non-target organisms. Genetically modified (GM) crops expressing insecticidal Bacillus thuringiensis (Bt) proteins provided a technology improvement on pest management that reduced the dependence on chemical insecticides (Phipps and Park, 2002; James, 2009; Areal and Riesgo, 2015). However, wide adoption of Bt trait technology has resulted in field-evolved resistance (Tabashnik et al., 2009, 2013). Consequently, new pest control strategies to overcome these obstacles are urgently required.

A promising alternative insect pest control strategy is the use of double-stranded RNAs (dsRNAs) to knock-down essential pest genes by triggering the conserved eukaryotic RNA interference (RNAi) pathway (Huvenne and Smaggle, 2010; Xue et al., 2012). Upon entry of gene-specific dsRNA into the cell of the intended pest organism, the expression of the targeted endogenous gene transcript will be knocked down post-transcriptionally and can result in insect mortality. Major advantages of this new approach are the rapid biological degradation of dsRNA in the environment and the sequence-specific nature of RNAi, allowing selective pest control that protects non-target organisms like pollinators or predatory insects (Dubelman et al., 2014; Albright et al., 2017; Parker et al., 2019). Baum et al. (2007) demonstrated that transgenic corn expressing a vacuolar ATPase subunit showed protection against feeding damage by Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae) infestation. Recent examples of successful host-induced gene silencing (HIGS) applications include potato plants resistant to Colorado potato beetles (Zhang et al., 2015), as well as resistant maize plants silencing additional RNAi targets of D. v. virgifera (Hu et al., 2016) (reviewed in Koch and Kogel, 2014). The first commercialized transgenic event that uses HIGS for pest control, jointly developed by Monsanto and Dow AgroSciences, was approved by the three US regulatory agencies (EPA, FDA, and USDA). The combined-trait product known as SmartStax PRO® expresses both Bt toxins and dsRNA targeting the D. v. virgifera Sf97 gene (Head et al., 2017). As RNAi is a very active research area and an emerging pest management tool with potential for plant protection, it is just a question of time before SmartStax Pro and other applied RNA suppression products will appear on the market.

A key step in RNAi based pest control is the identification of novel bioinsecticide targets, a challenging task, as many pests lack genomic data and are often not easy to rear under laboratory conditions (Vogel et al., 2014). Performing large-scale RNAi pre-screens in a model organism was often proposed as a solution for target-site validation in pest insects (Alves et al., 2010; Knorr et al., 2013; Ulrich et al., 2015). Recently, a high throughput RNAi screen functionally analyzed 5000 genes of the model beetle Tribolium castaneum, revealing a panel of 100 lethal target genes that caused ≥ 90% mortality (Dönitz et al., 2014; Ulrich et al., 2015). A selection of these lethal T. castaneum RNAi targets was used to test for efficacy of orthologous genes in D. v. virgifera and the oilseed rape pest Brassicogthes aeneus (previously known as Melligethes aeneus). This approach identified the highly active target genes DNA-directed RNA polymerase II subunit (RPII-140), dre4 (homolog of SPT16), and ncm or nucampholin (homolog of CWC22) (Knorr et al., 2018). These sensitive RNAi targets encode polypeptides with essential functions at different steps of transcription and splicing. More specifically, dre4 is part of the FACT (facilitates chromatin transactions) complex that acts as a general chromatin regulator (Belotserkovskaya and Reinberg, 2004; Yang et al., 2016). The core splicing factor ncm is directing eukaryotic translation initiation factor 4A3 (eIF4AIII) into the exon junction complex (EJC) (Barbosa et al., 2012) and RPII-140 catalyzes the transcription of DNA into RNA (Falkenburg et al., 1987).

The primary goal of the current work was to expand on these potent T. castaneum RNAi targets by silencing functionally connected genes. Target gene selection was based on a knowledge based approach supported by functional Protein-Protein Interaction (PPI) networks generated by the STRING search tool (Mering et al., 2005; Szklarczyk et al., 2015) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway information (Ogata et al., 1999). We screened 30 genes in T. castaneum and identified some potent novel RNAi targets that were active in both larval and adult stage. In addition, we evaluated if the newly discovered RNAi targets could be leveraged to the economically important pest species, the Western Corn Rootworm (WCR) D. v. virgifera, a devastating pest of maize in the US Corn Belt, and the pollen beetle B. aeneus, a key pest of oilseed rape. For both of these difficult-to-work with agricultural pest insects we identified novel RNAi target genes that caused more than 90% mortality, suggesting that these genes may be considered as potential RNAi target for RNA-based management.

**MATERIALS AND METHODS**

**Insect Rearing**

*Tribolium castaneum* (San Bernardino strain) were maintained on wholegrain flour as described previously (Knorr et al., 2009). Adult *B. aeneus* were collected from flowering *Brassica napus* fields and reared on greenhouse-grown rape plants in a climate chamber at 24/18°C (day/night), 70% relative humidity and a 16:8 (light:dark) photoperiod. Non-diapausing *D. v. virgifera* eggs (Crop Characteristics, Inc. Farmington, MN) were washed from soil, followed by surface-sterilization with 10% formaldehyde and subsequent rinsing in water (Števo and Cagán, 2012). The eggs were hatched on artificial diet at 28°C, as described previously (Pleau et al., 2002; Števo and Cagán, 2012; Tan et al., 2016).

**Protein-Protein Interaction (PPI) Data**

The protein-protein interaction (PPI) data for dre4, ncm, and RPII-140 were imported from the open-source STRING (Search Tool for Recurring Instances of Neighboring Genes) v11.0 database (STRING, https://string-db.org/) (Szklarczyk et al., 2015).

**RNA Isolation and Complementary DNA Synthesis**

Total RNA was extracted from *T. castaneum* larvae and *B. aeneus* adults using Direct-zol™ RNA MiniPrep (Zymo Research, Irvine, California, USA) according to the manufacturer's instructions. cDNA was synthesized from 500 ng RNA using
the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA).

Total RNA from *D. v. virgifera* eggs, larvae, and adults was extracted using TRIzol (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. cDNA was synthesized from 1 µg of total RNA using a SuperScript III reverse transcription kit (Thermo Fisher Scientific, Waltham, MA).

### Identification of *T. castaneum* Gene Orthologs in *D. v. virgifera* and *B. aeneus*

The *T. castaneum* genome sequence ver.Tcas5.2 was used for the identification of ortholog proteins of the transcriptome of *B. aeneus* generated by Vogel et al. (2014) using NCBI BLASTP with an *E*-value of 0.01. The output was further filtered by a minimum protein identity of 50% as well as a protein coverage of at least 75% and lower than 125%. Resulting hits were ranked by score; redundant and overlapping sequences were removed. *D. v. virgifera* homologous were identified by performing TBLASTN searches with candidate protein coding sequences that were run against BLASTTable databases comprising the unassigned *D. v. virgifera* sequence reads or the assembled contigs. Significant hits to a *D. v. virgifera* sequence (defined as lower than <1 × 10−10 for contig homologies and better than *E*-value of <1 × 10−10 for unassigned sequence reads homologies) were verified using BLASTX against the NCBI non-redundant database. The results of this BLASTX search confirmed that the *D. v. virgifera* homolog candidate gene sequences identified in the TBLASTN search indeed comprised *D. v. virgifera* genes, or were the best hits to the non-*D. v. virgifera* candidate gene sequence present in the *D. v. virgifera* sequences. In the majority of cases, *T. castaneum* candidate genes that were annotated as encoding a protein, showed clear sequence homology to a sequence or sequences within the *D. v. virgifera* transcriptome. Sequencher™ v4.9 (Gene Codes Corporation, Ann Arbor, MI) was used in a few cases to assembled contigs that were partially-overlapping into longer contigs.

### dsRNA Synthesis

The selected dsRNA sequences were screened against genomic and transcriptomic data to avoid complete matches of more than 19 nt to a potential off-target genes. Gene-specific primers for PCR (Supplementary Table 1) amplification were designed using VectorNTI (Invitrogen, Carlsbad, CA) or Primer3 software and contained a T7 promoter sequence (5’-TATAACGACTCATAAGGAGA- 3’) at their 5’ ends. The dsRNAs were synthesized using Ambion MEGAscript T7 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s protocol and quantified on a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

### Injection bioassays

Dorsolateral injection of 150 nl dsRNA [250 ng/µl] into *T. castaneum* larvae and *B. aeneus* adults was conducted using a glass capillary and a micromanipulator M3301 (World Precision Instruments, Sarasota, FL) under a dissecting stereomicroscope (*n* = 12). Negative controls received an equivalent amount of water or dsRNA corresponding to the *Galleria mellonella* metalloproteinase inhibitor (IMPI) gene (not present in *B. aeneus*) (Supplementary Table 1). Injected *T. castaneum* larvae were kept on whole-grain flour, whereas *B. aeneus* beetles were supplied with dried pollen and wet tissues as water source. Survival rates were monitored and insects counted every 48 h for 10 days.

### Feeding bioassays

*Brassicogethes aeneus* and *D. v. virgifera* feeding bioassays were performed as described previously (Knorr et al., 2018). In brief, *B. aeneus* adults ingested dsRNA [500 ng/cm²] using artificial diet to ensure continuous uptake of dsRNA. Negative controls received an equivalent amount of water or dsRNA corresponding to the *G. mellonella* metalloproteinase inhibitor (IMPI) gene (Supplementary Table 1). The number of dead beetles was counted every 48 h for 10 days.

Neonate *D. v. virgifera* larvae were fed on artificial diet that was overlaid with dsRNA (500 ng/cm²) (Knorr et al., 2018). Water, 0.1X TE buffer and dsRNA targeting yellow fluorescent protein gene (YFP) were used as negative controls. The number of dead insects, and the weights of surviving insects were recorded after 9 days. Growth Inhibition (GI) was calculated based on the average weights of all controls, as follows: GI = [1 – (TWIT/TNIT)/(TWIBC/TNIBC)], where TWIT is the total weight of insects in the treatment, TNIT is the total number of insects in the treatment, TWIBC is the total weight of insects in the buffer control, and TNIBC is the total number of insects in the buffer control. To estimate the 50% lethal concentration (LC50) and the concentration that causes 50% growth inhibition (GI50) of active RNAi targets, 4-fold serial dilutions of dsRNAs were assayed. Values were calculated using log-logistic regression analysis in JMP® Pro from SAS Institute Inc (Supplementary Table 1).

### Statistical Analysis

Bioassays were analyzed using an analyses of variance (ANOVA), followed by a Bonferroni-Holm test with significance threshold of *p* < 0.05 using Daniel’s XL toolbox for Excel, version 7.3.4 68. Each experiment was compared to a control group and all experiments were conducted independently at least three times. LC50 (concentration at which 50% of the insects are dead) and GI50 (concentration that causes 50% growth inhibition or GI) values were calculated as described earlier (Knorr et al., 2018).

### RESULTS

#### Knockdown of Potential Target Genes in *T. castaneum* via Injection of dsRNA

The three most active RNAi targets RPI-140, Dre4, and *ncm* described in Knorr et al. (2018) are involved in pre- and post-transcriptional mRNA processing. We mined corresponding String PPI networks and KEGG pathway for potential RNAi targets and selected 30 genes for *T. castaneum* dsRNA injection assays: 18 genes coding for different RNA polymerase subunits...
Knorr et al. Novel Pest Control RNAi Targets

| Gene | Tcas NCBI ID | Tcas larva | Tcas ♀ adults | Tcas ♂ adults | Dvir larvae | Baen adults |
|------|-------------|------------|---------------|---------------|-------------|-------------|
| 1    | RPI-1       | XM_965825  | 82.22 ± 1.15  | 6.67 ± 0.99   | 53.33 ± 2.98 | 79.41 ± 4.8  | n.d.        |
| 2    | RPI-12      | XM_962276  | 64.44 ± 1.52  | 13.33 ± 0.99 | 4.44 ± 0.57  | n.d.        | n.d.        |
| 3    | RPIin.d.135 | XM_967590  | 77.78 ± 0.57  | 64.44 ± 1.52  | 73.33 ± 2.63 | n.d.        | n.d.        |
| 4    | RPI-10      | NM_001143916 | 97.78 ± 0.57   | 100 ± 0       | 100 ± 0      | n.d.        | n.d.        |
| 5    | RPI-11      | XM_961832  | 91.11 ± 0.57  | 100 ± 0       | 97.78 ± 0.57 | n.d.        | n.d.        |
| 6    | RPI-15      | XM_961993  | 77.78 ± 1.52  | 100 ± 0       | 82.22 ± 0.57 | 56.17 ± 8.31 | n.d.        |
| 7    | RPI-16      | XM_961801  | 100 ± 0       | 100 ± 0       | 77.78 ± 0.57 | n.d.        | n.d.        |
| 8    | RPI-18      | XM_970262  | 100 ± 0       | 93.33 ± 0.99  | 97.78 ± 0.57 | n.d.        | n.d.        |
| 9    | RPI-33      | XM_965542  | 100 ± 0       | 88.89 ± 1.15  | 82.22 ± 1.15 | 94.12 ± 2.15 | 20.00 ± 1.83 |
| 10   | RPI-215     | XM_968377  | 100 ± 0       | 100 ± 0       | 97.78 ± 0.57 | 79.13 ± 5.20 | 93.33 ± 1.05 |
| 11   | RPIII-1     | XM_963072  | 97.78 ± 0.57  | 77.78 ± 3.49  | 100 ± 0      | n.d.        | n.d.        |
| 12   | RPIII-4     | XM_963604  | 68.89 ± 1.52  | 71.11 ± 1.52  | 73.33 ± 0    | n.d.        | n.d.        |
| 13   | RPIII-6     | XM_966403  | 77.78 ± 0.57  | 77.78 ± 1.52  | 84.44 ± 1.52 | n.d.        | n.d.        |
| 14   | RPIII-25    | XM_968337  | 57.78 ± 2.50  | 33.33 ± 3.44  | 44.44 ± 2.50 | n.d.        | n.d.        |
| 15   | RPIII-32    | XM_964761  | 55.56 ± 1.15  | 17.78 ± 2.07  | 93.33 ± 0.99 | n.d.        | n.d.        |
| 16   | RPIII-62    | XM_970507  | 64.44 ± 1.52  | 75.56 ± 1.52  | 93.33 ± 1.72 | n.d.        | n.d.        |
| 17   | RPIII-80    | XM_968935  | 75.56 ± 2.07  | 68.89 ± 1.52  | 84.44 ± 1.52 | n.d.        | n.d.        |
| 18   | RPIII-128   | XM_961456  | 68.89 ± 3.76  | 88.89 ± 1.52  | 95.56 ± 0.57 | 5.88 ± 0    | 46.67 ± 3.80 |
| 19   | Cdk8        | XM_966689  | 94.44 ± 1.60  | n.d.          | n.d.        | n.d.        | n.d.        |
| 20   | Cwc25       | XM_967699  | 50.00 ± 0     | n.d.          | 3.33 ± 3.33  | n.d.        | n.d.        |
| 21   | elf4AIII    | XM_970418  | 88.89 ± 0.80  | n.d.          | n.d.        | n.d.        | n.d.        |
| 22   | elf3        | XM_967756  | 94.44 ± 1.60  | n.d.          | n.d.        | n.d.        | n.d.        |
| 23   | Prp8        | XM_961838  | 88.89 ± 2.12  | n.d.          | 68.90 ± 8.63 | n.d.        | n.d.        |
| 24   | Prp16       | XM_966423  | 80.56 ± 2.12  | n.d.          | n.d.        | n.d.        | n.d.        |
| 25   | Prp19       | XM_964912  | 88.89 ± 1.60  | n.d.          | 20.58 ± 5.51 | 86.67 ± 2.11 | n.d.        |
| 26   | SF3B4       | XM_963027  | 33.33 ± 4.17  | n.d.          | n.d.        | n.d.        | n.d.        |
| 27   | Spt5        | XM_966005  | 100 ± 0       | n.d.          | 98.53 ± 1.47 | 50.00 ± 3.65 | n.d.        |
| 28   | Spt6        | XM_962996  | 100 ± 0       | n.d.          | 89.51 ± 4.35 | 40.00 ± 1.83 | n.d.        |
| 29   | SSU72       | XM_963870  | 22.22 ± 1.60  | n.d.          | n.d.        | n.d.        | n.d.        |
| 30   | TFIIID      | XM_961281  | 38.89 ± 2.12  | n.d.          | n.d.        | n.d.        | n.d.        |

T. castaneum gene names and NCBI RefSeq are presented. T. castaneum larvae and adults as well as B. aeneus adults were injected with 150 nl of 250 ng/µl dsRNA and assessed 10 dpi. D. v. virgifera larvae were fed dsRNA (800 ng/cm²) in diet overlay bioassays for 9 days.

Raw bioassay data are included Supplementary Table 1. n.d., not determined.

(RP genes) and 12 genes involved in various transcriptional processes (non-RP genes) (Table 1, Figure 1).

Homologs of the selected 30 genes were identified via BLAST (tblastn) homology searches using Drosophila melanogaster protein isoforms (see Supplementary Table 1 for GenBank IDs) as the queries. To test whether dsRNA targeting these genes may lead to lethal phenotypes in T. castaneum, we designed dsRNA constructs targeting a segment of the open reading frame and applied the dsRNA via injection into the larval hemocoel. Significant mortality of >50% was observed onset on the 4th day after injection of ~150 nl of 250 ng/µl dsRNA targeting the four genes cdk8, Prp8, Spt5, and Spt6 (Figure 2A). The detected mortality rates were significantly higher compared to the non-target IMPI dsRNA. Significant mortality of over 70% was caused by 19 out of the 30 applied dsRNA constructs 10 days post injection (dpi). The highest mortality of 100% was recorded for the six genes Spt5, Spt6, RPII-16, RPIII-18, RPII-33, and RPII-215 (Table 1, Figure 2). In addition, silencing RNA polymerase subunits (RPs) caused over 50% larval mortality in all 18 treatments 10 dpi (Table 1, Figure 2B).

We also analyzed the potential lethal effect of the 18 RP dsRNA constructs in T. castaneum female and male adults. Significant mortality rates of over 50% were observed for 14 female and 16 male dsRNA RP constructs 10 dpi (Table 1, Figure 3), including six female and eight treatments that caused over 90% mortality, respectively (Table 1, Figure 3). RPII-215 was one of the most efficacious RNA targets, causing over 75% female mortality, 50% and 97% mortality in males 6 dpi. Only knockdown of RPI-1 and RPIII-32 resulted in considerably lower female mortality rates of ~7 and ~18%, respectively. Whereas, in males silencing of RPI-1 led to >50% and RPIII-32 to >90% lethality.
Knockdown of Potential Target Genes in D. v. virgifera Larvae Upon Feeding

The four most lethal target genes Sp5, Sp6, RPII-215, and RPII-33, as well as the six target genes Prp8, Prp19, Cwc25, RPI-1, RPII-15, and RPII-128 with moderate activity, were selected from the T. castaneum screen for D. v. virgifera knockdown experiments. Diet overlay bioassays using 500 ng/cm² dsRNA identified seven out of the 10 dsRNA constructs that caused significant mortality of over 50%. Seven of the tested target genes, caused over 70% mortality (Figure 4A), including dsRNAs targeting Sp5, Sp6, and RPII-33 transcripts, which resulted in the highest mortality rates of 99, 90, and 88%, respectively. In addition, RNAi treated groups showed significant growth inhibition in D. v. virgifera ranging from 0.72 to 0.99 in all treatments, except for the Cwc25 treatment (Figure 4B, Supplementary Table 1).

In addition, the median lethal concentration (LC₅₀) and median growth inhibition (GI₅₀) values for dsRNA treatments that caused > 60% mortality were further investigated. A dilution series of dsRNA tested in diet-overlay bioassays led to LC₅₀ values that ranged from 4.33 to 54.26 ng/cm² (Table 2). The majority of dsRNAs tested resulted in LC₅₀...
FIGURE 2 | Mortality rates of *T. castaneum* larvae after knockdown. Injection of 150 nl of dsRNA [250 ng/µl] targeting genes involved in the process of transcription (A) and different RNA polymerase subsunits (B) into *T. castaneum* larvae. Mortality rates were tracked every 48 h for 10 days. The *G. mellonella* gene IMPI was used as negative control. The data represent three biological replicates (*n* = 15 for each replicate). Asterisks show statistical difference (*p* < 0.001). Green stars represents selected *D. v. virgifera* and yellow *B. aeneus* orthologs. The results were analyzed with analysis of variance (ANOVA) (Supplementary Table 1).
values of <20 ng/cm², in particular, the LC50 values of Spt5 and Spt6 were 4.33 and 4.83 ng/cm², respectively. The GI50 values ranged from 1.38 to 27.92 ng/cm² (Table 2), whereas Spt5 and Prp8 showed the lowest values of 1.38 and 2.40 ng/cm².

Knockdown of Potential Target Genes in *B. aeneus* Adults via Injection of dsRNA

The four most lethal target genes Spt5, Spt6, RPII-215, and RPII-33, as well as Prp19 and RPIII-128 as moderately potent target genes were selected from the *T. castaneum* screen for *B. aeneus* adults. Mortality rates were tracked every 48 h for 10 days. The *G. mellonella* gene IMPI was used as a negative control. The data represent three biological replicates (*n* = 15 for each replicate). Asterisks show statistical difference (*p* < 0.001). The results were analyzed with analysis of variance (ANOVA) (Supplementary Table 1).

**FIGURE 3** Mortality rates of *T. castaneum* adults after knockdown. Injection of 150 nl of dsRNA (250 ng/µl) targeting different RNA polymerase subunits into *T. castaneum* (A) female and (B) male adults. Mortality rates were tracked every 48 h for 10 days. The *G. mellonella* gene IMPI was used as negative control. The data represent three biological replicates (*n* = 15 for each replicate). Asterisks show statistical difference (*p* < 0.001). The results were analyzed with analysis of variance (ANOVA) (Supplementary Table 1).
Chemical insecticides are still the major control method for insect pests, but widespread resistance development and environmental concerns like non-target toxicity create the need for innovative and sustainable pest control methods (Tabashnik, 1994; Budzinski and Couderchet, 2018). RNAi-based crop protection is a new-generation technology for highly efficient, environmentally sound biopesticides that target insect pests with great specificity. To date, insecticidal RNAi target genes have been identified in key biological systems of the insect physiology like digestion, defense mechanisms, vesicular trafficking, and detoxification (Mao et al., 2007; Bautista et al., 2009; Kim et al., 2015; Kola et al., 2015; Head et al., 2017; Knorr et al., 2018; Cooper et al., 2019). A major challenge for the development of RNAi applications, however, is the identification of novel sensitive target genes, in particular in non-model organisms that often lack genomic data or laboratory colonies (Vogel et al., 2014). Some researchers take advantage of known RNAi-sensitive genes in model organisms and perform targeted gene mining strategies to verify the orthologous RNAi target in other insects (Pitino et al., 2011; Wang et al., 2011; Zhao et al., 2011; Zhu et al., 2011; Li et al., 2013; Rodrigues et al., 2017; Mogilicherla et al., 2018; Koo et al., 2020). Recently, a knowledge-based approach demonstrated that the spectrum of known lethal RNAi targets could be expanded by silencing genes that are functionally connected (Bingsohn et al., 2017).

In this study, we mined the biological networks of RNA transcription and RNA processing, as silencing of genes with essential functions in these processes have demonstrated high mortality rates in three different coleopteran pests (Knorr et al., 2018). It has been shown that miRNA knockdown efficiency is not necessarily positively related to high mortality rates. Several uncontrollable factors can influence the RNAi response, like protein/mRNA half-life, gene function redundancy or the physiological importance of the targeted gene function (Schwinghammer et al., 2011; Li et al., 2013; Rinkevich and Scott, 2013; Bingsohn et al., 2017; Raje et al., 2018; Howell et al., 2020; Koo et al., 2020). Thus, we focused on organism-level analysis and did not consider gene expression as a key criterion for target selection. Out of 30 genes assayed in T. castaneum, 19 genes caused significant mortality of over 70%, including 11 target genes that lead to >90% mortality at day six post injection. Furthermore, silencing of RNA polymerase subunits in T. castaneum larvae caused significant mortality of at least 50% mortality in all 18 treatments and even 100% when knocking down RPII-215, RPII-33, RPII-18, and RPII-16. RNA polymerases subunits are also sensitive targets in adult beetles, both female and males, as knock down of seven subunits (RPII-1, RPII-10 RPII-11 RPII-15 RPII-16 RPII-215, and RPII-1) led to 100% mortality 10 days after dsRNA injection. Taken together, 36% of the silenced transcription- and splicing-related genes were identified as highly effective RNAi targets (≥90%, mortality). In comparison, only two percent of 5000 randomly screened Tribolium genes in

**DISCUSSION**

Insect damage to crops along with climate change and the loss of arable land dedicated to agriculture threaten the ability to meet the food demand of a rapidly growing global population (FAO, 2017). Chemical insecticides are still the major control method for insect pests, but widespread resistance development and environmental concerns like non-target toxicity create the need for innovative and sustainable pest control methods (Tabashnik, 1994; Budzinski and Couderchet, 2018). RNAi-based crop protection is a new-generation technology for highly efficient, environmentally sound biopesticides that target insect pests with great specificity. To date, insecticidal RNAi target genes have been identified in key biological systems of the insect physiology like digestion, defense mechanisms, vesicular trafficking, and detoxification (Mao et al., 2007; Bautista et al., 2009; Kim et al., 2015; Kola et al., 2015; Head et al., 2017; Knorr et al., 2018; Cooper et al., 2019). A major challenge for the development of RNAi applications, however, is the identification of novel sensitive target genes, in particular in non-model organisms that often lack genomic data or laboratory colonies (Vogel et al., 2014). Some researchers take advantage of known RNAi-sensitive genes in model organisms and perform targeted gene mining strategies to verify the orthologous RNAi target in other insects (Pitino et al., 2011; Wang et al., 2011; Zhao et al., 2011; Zhu et al., 2011; Li et al., 2013; Rodrigues et al., 2017; Mogilicherla et al., 2018; Koo et al., 2020). Recently, a knowledge-based approach demonstrated that the spectrum of known lethal RNAi targets could be expanded by silencing genes that are functionally connected (Bingsohn et al., 2017).

In this study, we mined the biological networks of RNA transcription and RNA processing, as silencing of genes with essential functions in these processes have demonstrated high mortality rates in three different coleopteran pests (Knorr et al., 2018). It has been shown that miRNA knockdown efficiency is not necessarily positively related to high mortality rates. Several uncontrollable factors can influence the RNAi response, like protein/mRNA half-life, gene function redundancy or the physiological importance of the targeted gene function (Schwinghammer et al., 2011; Li et al., 2013; Rinkevich and Scott, 2013; Bingsohn et al., 2017; Raje et al., 2018; Howell et al., 2020; Koo et al., 2020). Thus, we focused on organism-level analysis and did not consider gene expression as a key criterion for target selection. Out of 30 genes assayed in T. castaneum, 19 genes caused significant mortality of over 70%, including 11 target genes that lead to >90% mortality at day six post injection. Furthermore, silencing of RNA polymerase subunits in T. castaneum larvae caused significant mortality of at least 50% mortality in all 18 treatments and even 100% when knocking down RPII-215, RPII-33, RPII-18, and RPII-16. RNA polymerases subunits are also sensitive targets in adult beetles, both female and males, as knock down of seven subunits (RPII-1, RPII-10 RPII-11 RPII-15 RPII-16 RPII-215, and RPII-1) led to 100% mortality 10 days after dsRNA injection. Taken together, 36% of the silenced transcription- and splicing-related genes were identified as highly effective RNAi targets (≥90%, mortality). In comparison, only two percent of 5000 randomly screened Tribolium genes in

**TABLE 2** | LC50 and GI50 for RPI-1, RPII-33, RPII-215, Prp8, Spt5, and Spt6 in larval D. v. virgifera diet-based bioassay 9 dpf.

| dsRNA construct | LC50 (ng/cm²) | Range (ng/cm²) | GI50 |
|------------------|--------------|----------------|------|
| RPI-1            | 42.79        | 26.79–88.33    | 17.64 |
| RPII-33          | 13.57        | 26.68–15.86    | 15.86 |
| RPII-215         | 54.26        | 39.45–39.45    | 27.92 |
| Prp8             | 19.25        | 11.09–33.40    | 2.40  |
| Spt5             | 4.33         | 2.29–8.17      | 1.38  |
| Spt6             | 4.84         | 2.49–9.39      | 3.68  |
Knorr et al., 2014; Williams et al., 2010; Hervé and Cortesero, 2012; Bass and Field, 2018; Knorr et al., 2018; Willow et al., 2020a,b). The decreased sensitivity led but might. These results demonstrate that a knowledge-based approach can results in a higher probability of identifying efficient RNAi targets than high-throughput experiments.

To determine if the T. castaneum RNAi-sensitive targets could have a similar effect in other coleopteran pests, we identified orthologous sequences in D. v. virgifera and B. aeneus using transcriptome data mining. Significant growth inhibition was detected for all ten dsRNA constructs that were tested via artificial diet assays using neonate WCR larvae, out of which seven also caused significant mortality of over 50%. Silencing of the transcription elongation factors Spt5 and Spt6, two of the most active T. castaneum RNAi targets in this study, also caused high mortality rates of 99 and 90% in D. v. virgifera, respectively, and the lowest LC50 values of approximately 4 ng/cm². Due to the lack of B. aeneus lab-colonies, pollen beetle experiments were limited to field collected beetles and focused on injection assays of six active T. castaneum target genes, namely Spt5, Spt6, Prp19, RPII-215, RPII-33, and RIII-128. Knockdown of Prp19 and RPII-215 led to high mortality rates of 87 and 94%, respectively. Silencing of RPII-215, resulted in the most rapid response of 50% mortality 6 days after treatment. Delivering dsRNA via injection is, of course, not a practical technique for RNAi field applications (Huvenne and Smagghe, 2010; Xue et al., 2012). Environmental RNAi response in B. aeneus was previously reported (Knorr et al., 2018; Willow et al., 2020a,b) but might lead to variable knockdown efficiency, which, in this initial study, we wanted to avoid by applying, standardized dsRNA doses to the beetles. Oral uptake of dsRNA might have different dynamics than injection and thus, lethal activity needs to be verified in feeding experiments before implementing results of this study in the greenhouse and the field.

Our study demonstrated a high sensitivity to RNAi of genes involved in RNA transcription and mRNA splicing, providing opportunities to screen for additional targets in these functional classes. In particular, silencing genes that are involved in RNAPII mediated mRNA transcription resulted in severe mortality, revealing a prime source for highly active RNAi targets. The Suppresser of Ty (SPT) genes Spt5 and Spt6, most active targets in T. castaneum and D. v. virgifera, encode polypeptides with essential functions in early transcription elongation. Spt5 forms with Spt4 the conserved heterodimer DSIF (DRB sensitivity-inducing factor) that regulates processive RNAPII transcription elongation (Price, 2000; Winston, 2001; Jennings et al., 2004; Decker, 2020). The histone chaperone Spt6 assembles histones into nucleosomes and is able to escalate the RNAPII elongation rate (Ardehali et al., 2009; McDonald et al., 2010).

Synthetic agrochemicals have traditionally been the method of choice for pollen beetle control in oil seed rape (OSR), but their excessive application, particularly pesticides of the pyrethroid class, has led to the development of populations with high level of resistance (Zimmer et al., 2014). The decreased sensitivity led to the introduction of non-cross-resistant alternative insecticides, such as neonicotinoids. However, adverse effects on non-target organisms like bees and other pollinators resulted in the ban of several neonicotinoids (Bass and Field, 2018). RNAi mediated pollen beetle control via spray-induced gene silencing (SIGS), whereby target-specific dsRNA is sprayed onto host plants, represents a promising, ecologically safe alternative. Foliar applications of naked dsRNA have already shown to be active against different Coleopteran species (e.g., de Andrade and Hunter, 2016; San Miguel and Scott, 2016). But, OSR Brassica napus crops have a short vulnerability window of only a few weeks, as agronomic crop loss is only caused by adults feeding from flower buds (Williams et al., 2010; Hervé and Cortesero, 2016). Thus, dose response studies need to identify most sensitive target genes in order to develop fast acting dsRNA applications. Formulation of dsRNA might further improve potential HIGS applications. dsRNA formulated with nanoparticles like BioClay or Guanyle Polyomers demonstrated increased RNAi efficiency.
by overcoming problems like stability or cell uptake (Mitter et al., 2017; Christiaens et al., 2018; Parsons et al., 2018). Behavioral studies have shown a preference of *B. aeneus* for turnip rape (*Brassica rapa*) over oilseed rape, which can be used for a push-pull strategy (Vinatier et al., 2012; Hervé and Cortesero, 2016; Skellern and Cook, 2018). Push-pull strategies involve integration of stimuli that deter insect pests away from their main crop (push), while simultaneously luring them to trap plants (pull) (Pyke et al., 1987; Cook et al., 2007). Combining HIGS with trap cropping would reduce the treated field area, leading to a more effective outcome of the dsRNA product.

RNAi represents a promising alternative for ecologically sustainable agricultural insect pest. Species-specific dsRNA based insecticides might complement other biosafe measures (e.g., conservation biocontrol) for managing agricultural pests and could become a part of integrated pest management (IPM) approaches. RNAi pre-screening in a model insect significantly increased the probability of identifying high levels of efficacious RNAi targets in pest species that lack genomic information. However, our data highlights the need to validate candidate targets in the insect species of concern because there is not a known correlation of gene RNAi sensitivity across insect species. Of the analyzed genes, 30–40% caused over 90% mortality in *D. v. virgifera* and *B. aeneus*. In addition, knockdown of RPII-215 resulted in significant mortality in all three coleopteran species: 100% in *T. castaneum*, 93% in *B. aeneus* and 79% in *D. v. virgifera* assays. These findings suggest that the function of these selected genes might be conserved across coleopterans. However, silencing of Prp19 resulted in high mortality rates of 81% in *T. castaneum* and 87% *B. aeneus*, but not in *D. v. virgifera*. Knockdown of Spt5, Spt6, and RPII-33 in *T. castaneum* was 100% lethal in all three cases and also caused significant mortality rates of 99, 90, and 98%, respectively, in *D. v. virgifera*. Targeting RPII-33 in *B. aeneus* did not led to any significant mortality rates, but silencing of Spt5 and Spt6 did, albeit to a lesser extent.

To summarize, we demonstrate that a knowledge-based approach increased the efficiency in discovering potent RNAi targets in *Tribolium*. Furthermore, identified *T. castaneum* RNAi targets were also highly efficacious in two additional coleopteran pest species, *D. v. virgifera* and *B. aeneus*, representing promising candidates for RNAi-based pest control. Next steps should focus on greenhouse and field demonstration of RNAi candidates that might become a promising component in future IPM programs.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

**AUTHOR CONTRIBUTIONS**

EK, KN, and AV designed the study with input from other team members. MF, PG, and KA identified the *D. v. virgifera* target orthologs. AB identified the *B. aeneus* RNAi target orthologs. EK and LT designed and executed *T. castaneum* and *B. aeneus* experiments. MR and WL carried out *D. v. virgifera* diet bioassay. EK, EF, CG, AB, and KN interpreted the data. EK and KN composed the manuscript. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2021.715823/full#supplementary-material

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**Conflict of Interest:** PG, WL, and CG are employees of Corteva Agriscience. MF, KA, MR, EF, and KN are former employees of Corteva Agriscience.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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