PLOS ONE

RESEARCH ARTICLE

Host interactors of effector proteins of the lettuce downy mildew *Bremia lactucae* obtained by yeast two-hybrid screening

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Citation: Pelgrom AJE, Meisrimler C-N, Elberse J, Koorman T, Boxem M, Van den Ackerveken G (2020) Host interactors of effector proteins of the lettuce downy mildew *Bremia lactucae* obtained by yeast two-hybrid screening. PLoS ONE 15(5): e0226540. https://doi.org/10.1371/journal.pone.0226540

Editor: Mark Gijzen, Agriculture and Agri-Food Canada, CANADA

Received: December 1, 2019

Accepted: April 24, 2020

Published: May 12, 2020

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: GVdA was awarded grant #12683 by the Netherlands Organization for Scientific Research (https://www.nwo.nl/). MB was awarded grant 864.09.008 by the Netherlands Organization for Scientific Research (https://www.nwo.nl/).

Competing interests: The authors have declared that no competing interests exist.

Abstract

Plant pathogenic bacteria, fungi and oomycetes secrete effector proteins to manipulate host cell processes to establish a successful infection. Over the last decade the genomes and transcriptomes of many agriculturally important plant pathogens have been sequenced and vast candidate effector repertoires were identified using bioinformatic analyses. Elucidating the contribution of individual effectors to pathogenicity is the next major hurdle. To advance our understanding of the molecular mechanisms underlying lettuce susceptibility to the downy mildew *Bremia lactucae*, we mapped physical interactions between *B. lactucae* effectors and lettuce candidate target proteins. Using a lettuce cDNA library-based yeast-two-hybrid system, 61 protein-protein interactions were identified, involving 21 *B. lactucae* effectors and 46 unique lettuce proteins. The top ten interactors based on the number of independent colonies identified in the Y2H and two interactors that belong to gene families involved in plant immunity, were further characterized. We determined the subcellular localization of the fluorescently tagged lettuce proteins and their interacting effectors. Importantly, relocalization of effectors or their interactors to the nucleus was observed for four protein-pairs upon their co-expression, supporting their interaction *in planta*.

Introduction

Plant pathogenic bacteria, fungi and oomycetes deploy effector proteins to manipulate host cell processes. Importantly, effectors serve to suppress and circumvent plant immune responses. Basal host defense responses are activated upon recognition of ubiquitously present microbe-associated molecular patterns (MAMPs) by plant pattern recognition receptors (PRRs). Evolutionary adapted pathogens deploy effectors to suppress this pattern-triggered immunity (PTI). Specialized intracellular nucleotide-binding and leucine-rich repeat receptors (NLRs) recognize host translocated effectors, or the perturbations effectors induce on host

proteins, resulting in the activation of effector-triggered immunity (ETI). In turn, ETI can be counteracted by other effectors leading to a state of effector-triggered susceptibility (ETS) [1].

Fungi and oomycetes secrete apoplastic effectors that operate at the host-pathogen interface, and host-translocated effectors that act intracellularly in the host. The genomes of different pathogens encode for extensive candidate effector sets, which have specific characteristics based on their origin. Fungal genomes encode e.g. small apoplastic cysteine-rich proteins [2], plant pathogenic downy mildews and *Phytophthora* species express host-translocated Crinklers and RXLR effectors [3–6] whereas plant pathogenic Gram-negative bacteria, e.g. *Pseudomonas syringae*, inject type III effectors into host cells [7]. At present, the major challenge lies in elucidating the contribution of individual effectors to the infection process through the identification of candidate plant targets and analysis of the molecular mechanisms that contribute to disease susceptibility.

To systematically identify candidate effector targets in *Arabidopsis thaliana*, physical interactions between *Arabidopsis* proteins and effector proteins of the bacterium *P. syringae*, the obligate biotrophic oomycete *Hyaloperonospora arabidopsidis*, and the obligate biotrophic ascomycete *Golovinomyces orontii* were mapped using a systematic yeast-two-hybrid (Y2H) screening of ~8000 Arabidopsis ORFs [8,9]. Interactions between 123 effectors and 178 Arabidopsis proteins were found. Nine Arabidopsis proteins interacted with effectors from all three pathogens, whereas another 24 proteins interacted with effectors from two of the three pathogens. Arabidopsis proteins that interacted with effectors from multiple pathogens were proposed to function as cellular hubs on which effectors of different microbial kingdoms converge to effectively undermine plant immune responses [8]. Disease assays with one or more pathogens were performed on Arabidopsis insertion mutants corresponding to 124 interactors and an altered susceptibility phenotype was observed in mutant lines for 63 interactors. Susceptibility phenotypes were more frequently observed in mutant lines corresponding to interactors that interacted with multiple effectors [9].

The obligate biotrophic oomycete *Bremia lactucae*, the causal agent of downy mildew disease of lettuce, is a major problem in lettuce production worldwide. Consequently, a multitude of genetic studies have been carried out resulting in the identification of over 50 genes mediating resistance to *B. lactucae* in lettuce [10]. On the pathogen side, research efforts have led to the identification and cloning of one Crinkler and 49 *B. lactucae* RXLR-like effectors in *B. lactucae* isolate Bl:24 [11,12]. Recently, 161 candidate secreted RxLR effectors were identified in the genome of *B. lactucae* isolate SF5 [13]. To gain insight into the molecular mechanisms underlying lettuce susceptibility to *B. lactucae* infection, we here describe the identification of interactions between 21 *B. lactucae* effectors and 46 unique lettuce proteins, uncovered using the Y2H system. The subcellular localization of twelve interactors, selected on Y2H performance and predicted biological function, as well as their interacting effectors was visualized by confocal fluorescence microscopy. Upon co-expression of these effectors and their lettuce interactors in *Nicotiana benthamiana*, relocalization of the effector or target to the nucleus was observed in four instances, providing additional evidence that these interactions occur *in planta* and the Y2H-identified interactors are candidate effector targets.

Materials & methods

Generation of the Y2H prey library and bait constructs

A *Lactuca sativa* cv. Olof cDNA library was constructed using Invitrogen Custom Services (Invitrogen, Carlsbad, CA). Briefly, RNA was isolated using phenol/ chloroform extraction from mock-treated seedlings, *Bremia lactucae* isolate Bl:24 (compatible interaction) and isolate F703 (incompatible interaction)-infected seedlings at 3 days after inoculation (dpi), and

seedlings treated with the salicylic acid (SA) analog benzothiadiazole (BTH; 0.1 mg/ml) 24h after spraying. RNA originating from the different treatments, was mixed in equal amounts. A three-frame uncut oligo(dT)-primed cDNA library in pENTR222 was created from 2 mg RNA using Gateway cloning technology. The library was transferred into the yeast-two-hybrid destination vector pDEST22 to generate GAL4 activation domain (AD) lettuce fusion proteins. The pDEST22 library in *E. coli* strain DH10B originated from 22 x 10^6 colony forming units (cfu) with an average insert size of 1.1 kb.

B. lactucae effectors were amplified using cDNA from the sequence encoding the predicted signal peptide cleavage sites and new start codons were introduced. Gateway entry clones of *B. lactucae* effectors were recombined with the pDEST32 yeast-two-hybrid destination vector using LR clonase to generate GAL4 DNA binding domain (DBD) effector fusion proteins.

Yeast strains and transformation

To create competent yeast, cells were grown o/n in 250 ml YEPD at 28˚C and 200 rpm to an OD_{600} of 0.2–0.8. Cells were spun down at 500 rcf for 5 min, washed with 50 ml sterile ddH_2O, spun down again and washed with 50 ml TE/LiAc (100 mM LiAc, 10 mM Tris, 1 mM EDTA, pH 8.0). After a final centrifugation step, the yeast was resuspended in TE/LiAc to an OD_{600} of 50. For single construct transformation, 20 μl of competent yeast was gently mixed with 11 μl 10xTE (100 mM Tris, 10 mM EDTA, pH 8.0), 13 μl 1 M LiAc, 82 μl 60% PEG (MW 3,350), 20 μl salmon sperm DNA (Sigma #D1626; 2 mg/ml in TE, heated at 95˚C for 5 min and transferred to ice) and 200 ng plasmid. Reactions were incubated at 30˚C for 30 min, and then transferred to a water bath at 42˚C for 15 min. 1 ml ddH_2O was added to each transformation reaction, the tubes were spun down at 2300 rcf for 30 sec and the pellet was resuspensed in ddH_2O. Bait strains were plated on synthetic complete (Sc)–Leu medium and prey strains were plated on Sc–Trp medium. Colonies appeared after two to three days at 30˚C. For library transformation the protocol was scaled up to 3200 μl competent yeast cells and 90 μg plasmid DNA. Yeast colonies were harvested in YEPD medium + 20% glycerol and 1 ml aliquots with OD_{600} = 40 were frozen at -80˚C. The yeast prey library consisted of 1.1 x 10^6–1.5 x 10^6 individual colonies. Yeast strain Y8800 (genotype MATa trp1–901 leu2–3,112 ura3–52 his3– 200 gal4Δ gal80Δ cyh2R GAL1::HIS3@LYS2 GAL2::ADE2 GAL7::LacZ@met2) was used for prey and yeast strain Y8930 (genotype MATα trp1–901 leu2–3,112 ura3–52 his3–200 gal4Δ gal80Δ cyh2R GAL1::HIS3@LYS2 GAL2::ADE2 GAL7::LacZ@met2) was used for bait. Bait strains that grew on Sc–Leu–His plates in the absence of prey were considered auto-activating and discarded.

Library screening

The mating method was used for library screening [14]. Diploid yeast cells were plated on Sc–Leu–Trp–His + amp medium to identify interacting bait-prey combinations. Retransformation of bait and prey candidates was performed to confirm interactions. A detailed description can be found in S1 File.

Bioinformatic analysis of effectors and lettuce interactors

Lettuce prey gene models were extracted using a BlastN search against the lettuce genome [15]. Where necessary, incomplete gene models were corrected using the lettuce transcriptome [16] and prey sequencing data. Prey sequences with a stop codon within the first 50 amino acids (aa) after the GAL4-AD sequence were considered not in frame. Presence of signal peptides was predicted using SignalP4.1 [17] and transmembrane domains were identified with TMHMM [18,19] using the default parameters. Transmembrane domains identified in

effector sequences were validated using TOPCONS [20,21]. Domain prediction was performed using InterProScan5. The presence of importin-α dependent nuclear localization signals was predicted using cNLS Mapper [22]. WY domains were predicted using an HMM model [23] with an individual cut-off E-value of 0.001 for the best motif within an effector. Post-translational lipid modifications were predicted using GPS-Lipid/CSS-Palm [24,25] on effector sequences lacking the signal peptide.

L. sativa cv. Salinas coding sequences corresponding to genome v8 (Genome ID 28333) were downloaded from the CoGe platform (https://genomevolution.org/). Translated CDSs of interactors on the shortlist were searched using Hidden Markov Models for the presence of domains with *E*-value $1e^{-4}$ as the cut-off. In general, all gene models with the same Pfam domain were subsequently extracted to identify the corresponding gene families. All gene models belonging to the same gene family as the interactors on the shortlist were named collectively. Gene names are composed of the prefix '*Ls*' for *L. sativa* followed by a short abbreviation denoting the domain and a number. Genes were numbered according to their position on the lettuce linkage groups 1–9 to avoid issues due to the, often, complex orthologous relationship with Arabidopsis proteins. An overview of all the new nomenclature for gene models can be found in S2 File. A detailed description of the procedure per gene model can be found in S1 File.

Transient expression in *N. benthamiana*

Full-length prey sequences were amplified from lettuce cv. Olof cDNA or prey plasmid using primers listed in S1 Table and recombined in a modified pGemTEasy vector containing the pDONR201 Gateway recombination site (pGemTEasy^{mod}) using BP clonase. Bait and prey entry clones were further recombined in pUBN-YFP-DEST and pUBN-CFP-DEST (kind gift from Dr. Christopher Grefen; [26]) or with pB7WGY2 and pB7WGC2 [27] to create N-terminal YFP and CFP fusion proteins respectively using LR clonase and transformed in *Agrobacterium tumefaciens* strain C58C1 (pGV2260) with selection on rifampicin (50 μg/ml), carbenicillin (50 μg/ml) and spectinomycin (100 μg/ml). Leaves of four to five-week-old *N. benthamiana* plants were co-infiltrated with an *A. tumefaciens* strain carrying the P19 silencing suppressor [28] in combination with strains harbouring bait or prey fusion constructs resuspended in infiltration buffer (10 mM MES, 10 mM MgCl2, and 150 μM acetosyringone, pH 5.6) to an OD of 0.3 per *A. tumefaciens* strain.

Confocal fluorescence microscopy

Microscopy was performed at 2–3 days after *Agrobacterium*-infiltration using a Zeiss LSM 700 laser scanning microscope. Leaf sections were incubated in propidium iodide (PI) solution (5 mg/ml) for 7–10 min prior to imaging to stain the cell wall. If no cytoplasmic strands were observed over multiple samples and the fluorescence signal was only observed in the same plane as the propidium iodide signal, the localization was considered plasma membrane. Excitation of Cyan Fluorescent Protein (CFP), Yellow Fluorescent Protein (YFP) and PI was done at 405 nm, 488 nm and 555 nm respectively. Emitted light of CFP and YFP was captured using a 490–555 nm band-pass filter, whereas emitted light of PI was captured using a 560 nm long pass filter.

Immunoblotting

Leaves were harvested 2–3 days after infiltration with *Agrobacterium* suspensions, frozen in liquid nitrogen and stored until further processing at -80˚C. All subsequent steps were performed at 4˚C. Leaves were ground using mortar and pestle and proteins were extracted with

lysis buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.5 mM EDTA, 0.25% (v/v) Triton-X 100, 0.25% (v/v) Tween-20, 5 mM DTT, 4% (w/v) polyvinylpyrrolidone) supplemented with 2x protease inhibitor cocktail without EDTA (#11873580001, Roche) for 1 h at 4˚C in an Eppendorf ThermoMixer at 2000 rpm. Lysates were centrifuged for 15 min at 10 000 g. The supernatant was separated on Bolt pre-mixed cast gradient gels 4–12% with Bolt Mes-SDS running buffer (ThermoFisher Scientific) according to the manufacturer's instructions. Proteins were transferred for 100 min at 100 V to nitrocellulose membrane using Towbin buffer (25 mM Tris, 192 mM glycine, 20% (v/v) ethanol), detected with 1:2500 diluted anti-GFP HRP conjugated antibodies (Miltenyi Biotec Cat# 130-091-833, RRID:AB_247003, Lot no: 5180709500) using SuperSignal West Pico chemiluminescent substrate (ThermoFisher Scientific) and documented with a CCD-camera (ChemiDoc, Bio-Rad). Original Western blots are provided in Supplementary information S1 Raw images.

Results

Effector interaction screening identifies new and known target candidates

To investigate the molecular mechanisms underlying lettuce susceptibility to *B. lactucae* infection, we selected 46 previously identified candidate effectors from *B. lactucae* isolate Bl:24 [11,12,16] to be used as bait in Y2H screens (Table 1). Orthologs of 34 effectors were found in isolate SF5 [13]. Consistent with the biotrophic lifestyle of *B. lactucae*, the vast majority of these candidate effectors are classified as RXLR-like effectors; when we started the Y2H screen only a single Crinkler (*Bremia lactucae* Crinkler; BLC) had been discovered. The RXLR-like effectors can be subdivided in two groups: 1) effectors with an RXLR motif (BLR effectors) or RXLR-like motif such as GXLR (BLG effectors) and QXLR (BLQ effectors), and 2) effectors that show homology to RXLR effectors but lack an RXLR-like motif, the BLN (*Bremia lactucae* No RXLR motif) effectors. All of the BLN effectors and the majority of the effectors with an RXLR(-like) motif contain an EER(-like) motif. The effectors ranged in size from 65 to 814 amino acids with an average length of 248 aa.

Despite a lack of general sequence conservation between all effector proteins within a species, it was found that about 44% of the *P. infestans* effectors, 26% of *H. arabidopsidis* effectors and 18% of *Plasmopara halstedii* RxLR effectors contain WY-domains that form a structurally conserved α-helical fold [23,29,30]. Analysis of the 46 *B. lactucae* effectors revealed predicted WY domains in 7 effectors (15%) (Table 1). Among the potential WY domain-containing effectors are BLN06 and BLN08 that lack an RXLR motif, but are nevertheless recognized in specific lettuce lines [12,16], thereby demonstrating effector activity.

To identify lettuce proteins that are targeted by *B. lactucae* effectors a Y2H screen was performed. First, the coding sequences of the 46 *B. lactucae* effectors lacking the signal peptide-encoding part were cloned in frame with the GAL4-DBD in the bait vector pDEST32. Of the 46 bait constructs three showed auto-activation in the Y2H system. The remaining 43 effectors were used as baits in Y2H screens with a prey library composed of *L. sativa* cv. Olof complementary DNA (cDNA). To promote representation of lettuce transcripts relevant to host-pathogen interactions, lettuce seedlings were challenged with compatible or incompatible *B. lactucae* isolates or treated with a SA-analog before RNA extraction. To eliminate false positives, identified interactors from lettuce were carefully scrutinized. Firstly, coding sequences that were out of frame with the GAL4 activation domain-encoding sequence were discarded. Secondly, prey constructs that tested positive for autoactivation of the *HIS3* reporter in the absence of the corresponding bait construct were also removed. Thirdly, bait and prey plasmids were retransformed in yeast to test if the interaction could be confirmed. These selection steps resulted, predominantly, in the elimination of candidate effector interactors that were

Table 1. Overview of *Bremia lactucae* effectors and the number of interacting plant proteins by Y2H screening.

Crinklers								
Effector	*Protein length*	*LXLFLAK motif*		*HVLVXXP motif*		*# Interacting lettuce proteins*		
		Motif	*Start position*	*Motif*	*Start position*			
BLC01	198	LRLFLAK	49	HVLVVP	114	-		
RXLR-like effectors								
Effector	*Protein length*	*RXLR-like motif*		*EER-like motif*		*Predicted WY domains*	*TM domains*[a]	*# Interacting lettuce proteins*[b]
		Motif	*Start position*	*Motif*	*Start position*			
BLG01	336	GKLR	44	DER	57	-	-	-
BLG02	233	GRLR	44	DER	57	-	-	2
BLG03	243	GKLR	42	DER	55	-	-	1
BLN01	652	-	-	EER	48	Yes	-	7
BLN03	169	-	-	EER	52	-	Yes	1
BLN04	147	-	-	EER	59	-	Yes	3
BLN05	491	-	-	EER	50	-	-	-
BLN06	502	-	-	EER	51	Yes	-	-
BLN08	463	-	-	EER	72	Yes	-	-
BLQ01	79	QLLR	49	DEEQR	61	-	-	-
BLQ04	531	QILR	27	EER	54	-	-	2
BLR03	141	RFLR	48	EEER	59	-	-	2
BLR04	76	RELR	45	DIK	60	-	-	-
BLR05	97	RALR	32	DED	58	-	Yes	6
BLR07	253	RALR	47	EEER	68	-	-	-
BLR08	135	RLLR	38	-	-	-	Yes	4
BLR09	112	RRLR	37	EER	81	-	Yes	7
BLR11	463	RRLR	46	DESER	57	-	-	9
BLR12	123	RYLR	49	ELEK	61	-	Yes	1
BLR13	363	RRLR	44	EER	55	Yes	-	-
BLR14	75	RKLR	46	-	-	-	-	-
BLR15	102	RSLR	47	DEER	60	-	-	-
BLR16	98	RSLR	47	NDER	60	-	-	-
BLR17	282	RRLR	50	DAEK	64	-	-	-
BLR18	92	RALR	46	NEDR	55	-	-	2
BLR19	160	RLLR	45	DNNEER	54	-	-	AB
BLR20	130	RLLR	53	DEAD	69	Yes	-	1
BLR21	65	RILR	39	-	-	-	-	1
BLR22	185	RGLR	33	-	-	-	-	AB
BLR23	107	RSLR	57	DENR	62	-	-	-
BLR24	91	RSLR	55	ELEQ	74	-	-	-
BLR25	82	RALR	55	-	-	-	-	-
BLR26	187	RRLR	46	QNDER	59	-	-	2
BLR27	434	RQLR	40	-	-	-	-	4
BLR28	279	RRLR	49	-	-	-	-	1
BLR29	311	RMLR	35	EES	46	-	-	-
BLR30	101	RSLR	47	DEER	60	-	-	-
BLR31	126	RLLR	44	EER	56	-	-	-
BLR32	146	RLLR	44	DER	56	-	-	1
BLR33	270	RRLR	50	DER	64	-	-	-
BLR35	514	RSLR	47	EER	58	-	-	2

(*Continued*)

Table 1. (Continued)

BLR36	476	RALR	55	EER	68	Yes	-	-
BLR37	814	RRLR	42	-	-	Yes	-	AB
BLR38	260	RLLR	46	-	-	-	-	2
BLR40	145	RRLR	44	EER	56	-	-	-

[a] predicted using TMHMM Server v.2.0

[b] AB = autoactivating bait

https://doi.org/10.1371/journal.pone.0226540.t001

identified only once. In contrast, most candidate effector interactors identified in two or more yeast colonies proved reliable interactors. Therefore, all interactors that were identified only once were omitted from further analysis. Ultimately, the library screens and subsequent validation steps resulted in interactors for 21 effectors (46%) (Table 1) providing a set of 46 unique interacting lettuce proteins (Table 2). Using domain prediction and orthologous relationships to Arabidopsis proteins, nine lettuce proteins were given gene names (S1 and S2 Files).

Interactions between effectors and lettuce proteins were further classified according to the number of unique colonies corresponding to the same lettuce gene and the interaction strength based on reporter gene activation (Fig 1). Due to the stringent selection criteria, only two weak (activation of *HIS3* reporter only) interactions (3%) were identified, 25 interactions (41%) were classified as intermediate (activation of *HIS3* reporter in the presence of 2 mM 3AT) and 34 interactions (56%) were strong (activation of both the *HIS3* and *ADE2* reporters). The vast majority of interactions between effectors and lettuce interactors were highly specific: only eight preys (17%) interacted with multiple effectors. LsRTNLB05, a reticulon-like protein, stood out from other lettuce interactors due to its interaction with five effectors and a combined total of 104 yeast colonies (Table 2). The most yeast colonies, 50, within a single effector screen were identified with effector BLR18 for LsCSN5 that encodes COP9 signalosome subunit 5 (CSN5). However, LsCSN5 also displayed weak activation of the *HIS3* reporter in the presence of empty bait vector.

The most represented family of proteins in the screen is the prenylated rab acceptor (PRA1) family for which fragments of six members were identified and three members passed all selection criteria. *PRA1* genes encode small transmembrane proteins that localize to the secretory pathway in Arabidopsis and are proposed to play a role in vesicular trafficking in plants [31]. Also, they have previously been described as interactors of *G. orontii* effector candidates in Arabidopsis [9]. In lettuce the PRA1 protein family is composed of 17 members. The six Y2H identified PRA1 proteins were found in four clades of the phylogenetic tree (Fig 2A). To determine the specificity of the interactions between *B. lactucae* effectors and PRA1 proteins, a targeted Y2H assay was performed. All six identified prey constructs containing lettuce *PRA1* gene fragments were cotransformed with three interacting and one non-interacting *B. lactucae* effector in yeast and plated on selective medium of increasing stringency. The negative control BLR09 did not interact with any of the PRA1 members. The interactions between effectors BLR27, BLR32 and BLN01 with individual PRA1 proteins as identified in the library screens were confirmed. Furthermore, BLR27 and BLR32 interacted with more PRA1 proteins than determined by the library screen, indicating that the Y2H screening was not exhaustive and not all possible interactions were captured. In contrast, BLN01 interacted robustly with LsPRA1.B1 but only weakly with other PRA1 proteins (Fig 2B). Two of the three PRA1 proteins that passed all selection criteria belong to clade F. Interestingly, the Arabidopsis PRA1 proteins that interacted with *G. orontii* and *H. arabidopsidis* effectors also belong to clade F [9].

Table 2. Lettuce proteins identified in yeast-two-hybrid screens.

Lactuca ID[a]	Protein	Interacting effectors (# colonies)	Length (aa)	Signal peptide [b]	Transmembrane domains [c]	Domains/ family [d]
Lsa018798.1		BLG02 (7)	812	-	-	AAA ATPase, CDC48 family
Lsa022944.1	LsBPM3	BLG02 (9)	421	-	-	MATH/TRAF domain; BTB/POZ domain
Lsa042995.1	LsFER3	BLG03 (23), BLN01 (42)	270	-	-	Ferritin-like domain
Lsa013759.1		BLN01 (4)	219	-	79–98; 102–119; 139–161; 195–217	Prenylated rab acceptor PRA1 family
Lsa015767.1		BLN01 (3)	381	-	-	Zinc finger, RING/FYVE/PHD-type
Lsa019644.1		BLN01 (2)	408	-	372–394	-
Lsa021294.1		BLN01 (11)	347	-	-	WAT1-related protein
Lsa035501.1		BLN01 (3)	388	-	-	SANT/Myb domain
Lsa015570.1		BLN01 (8), BLR27 (6)	249	-	120–142; 152–174; 181–215	-
Lsa002122.1	LsRTNLB05	BLN03 (6), BLN04 (13), BLR05 (29), BLR08 (25), BLR09 (31)	296	-	129–147; 151–173; 217–239	Reticulon domain
Lsa008464.1		BLN04 (9), BLR05 (23), BLR08 (9), BLR09 (15)	191	1–24	114–136	-
Lsa040031.1		BLN04 (4), BLR05 (14), BLR08 (6), BLR09 (7)	497	-	467–486	NAC domain
Lsa031157.1		BLQ04 (2)	886	-	-	TATA element modulatory factor 1 DNA binding domain
Lsa004895.1		BLQ04 (4), BLR11 (4)	963	-	-	TATA element modulatory factor 1 DNA binding domain
Lsa009247.1		BLR03 (3)	418	-	-	CBS domain
Lsa038984.1		BLR03 (4)	338	-	-	E3 ubiquitin-protein ligase BOI-like family
Lsa002329.1		BLR05 (2)	137	-	111–133	Cytochrome b5-like heme/steroid binding domain
Lsa034832.1		BLR05 (2)	171	-	135–157	-
Lsa039137.1		BLR05 (3)	279	-	111–133; 159–181; 248–270	Uncharacterised protein family UPF0114
Lsa001248.1		BLR08 (2)	682	-	522–544; 614–636	Protein of unknown function DUF639
Lsa020711.1		BLR09 (2)	596	-	568–590	NAC domain
Lsa027896.1		BLR09 (3)	341	-	-	AIG1-type guanine nucleotide-binding (G) domain
Lsa033367.1		BLR09 (2)	552	-	530–547	CBS domain
Lsa040900.1		BLR09 (2)	576	-	-	Pentatricopeptide repeat domain
Lsa007441.1	LsHSP90-11	BLR11 (13)	698	-	-	Heat shock protein Hsp90 family
Lsa008910.1		BLR11 (2)	729	-	-	-
Lsa011626.1		BLR11 (2)	148	-	-	-
Lsa014576.1		BLR11 (3)	531	-	-	Ubiquitin system component Cue domain
Lsa017032.1		BLR11 (5)	671	-	-	-
Lsa038529.1		BLR11 (5)	561	-	-	Protein OBERON family
Lsa041640.1		BLR11 (3)	535	-	-	Serine/threonine/dual specificity protein kinase, catalytic domain
Lsa041732.1		BLR11 (4)	352	-	-	SH3 domain
Lsa011822.1	LsCSN5	BLR12 (3), BLR18 (50)	361	-	-	JAB1/MPN/MOV34 metalloenzyme domain
Lsa012741.1		BLR18 (7)	316	-	-	Thylakoid formation protein
Lsa006137.1	LsERF093	BLR20 (3)	290	-	-	AP2/ERF domain
Lsa027151.1		BLR21 (2)	207	-	-	Mitotic spindle checkpoint protein Mad2 family
Lsa010116.1		BLR26 (2)	177	-	18–40	Cytochrome b5-like heme/steroid binding domain

(*Continued*)

Table 2. (Continued)

Lactuca ID[a]	Protein	Interacting effectors (# colonies)	Length (aa)	Signal peptide [b]	Transmembrane domains [c]	Domains/ family [d]
Lsa044405.1		BLR26 (4)	214	-	23–45	Cytochrome b5-like heme/steroid binding domain
Lsa020102.1		BLR27 (7)	185	-	71–102; 122–156	Prenylated rab acceptor PRA1 family
Lsa021776.1		BLR27 (2)	274	-	-	RNA recognition motif domain
Lsa017864.1		BLR27 (7), BLR32 (3)	181	-	70–102; 117–136; 143–160	Prenylated rab acceptor PRA1 family
Lsa008980.1	LsDjA2	BLR28 (18)	420	-	-	Chaperone DnaJ domain
Lsa008956.1		BLR35 (5)	865	-	-	Kinesin motor domain; P-loop containing nucleoside triphosphate hydrolase domain
Lsa036902.1		BLR35 (9)	375	-	338–360	-
Lsa007018.1	LsFLX-like2	BLR38 (11)	382	-	-	-
Lsa043843.1		BLR38 (4)	429	-	-	Prephenate dehydratase domain

[a] according to the Lettuce Genome Resource http://lgr.genomecenter.ucdavis.edu/

[b] predicted using SignalP 4.1

[c] predicted using TMHMM Server v.2.0

[d] predicted using InterProScan

https://doi.org/10.1371/journal.pone.0226540.t002

Twelve plant interactors were selected for further exploration *in planta*, including the top ten ranked effector interactors by total number of yeast colonies. In addition, LsBPM3 and LsERF093 were chosen because of their predicted functions in targeting proteins for degradation and transcriptional regulation respectively, as will be described in more detail later.

To further explore the selected effector interactors, a closer look was taken at the prey plasmid-encoded gene fragments. DNA sequences obtained by Sanger sequencing using a vector specific forward primer were aligned to the corresponding lettuce coding sequences. A vector specific reverse primer could not be used due to the presence of poly(A)tails in prey inserts leading to slippage of the polymerase during sequencing. Therefore, information on the 3' end of inserts >800bp was not obtained. Lettuce gene fragments that included the start codon of the lettuce coding sequence (Fig 3) were detected for eight preys. Yeast clones of the remaining four preys only contained fragments that started downstream of the predicted start codon. The use of a cDNA library that contains both full-length coding sequences and gene fragments likely increased the number of identified protein-protein interactions.

Colocalization of effectors and their plant interactors

As proteins that are localized in the same subcellular compartment *in planta* are more likely to be true interactors, we set out to determine the subcellular localization of 15 *B. lactucae* effectors and 12 plant interactors using confocal fluorescence microscopy. The full-length coding sequences of the selected lettuce genes were cloned and fused downstream of *CFP*. Effector sequences were fused to *YFP*. The fusion proteins were produced in leaves of *Nicotiana benthamiana* using *Agrobacterium*-mediated transient expression. Immunodetection, using GFP-antibodies showed that for most proteins a fusion product of the expected size was produced by overexpression *in planta* (Figs 4 and 5). Detection of YFP-BLR18 showed a large fraction of free YFP and a minor fraction corresponding to the intact fusion protein, suggesting that the observed nucleocytoplasmic signal may result from free YFP. For other effector fusions that do not display an even nucleocytoplasmic localization pattern, it is likely that the

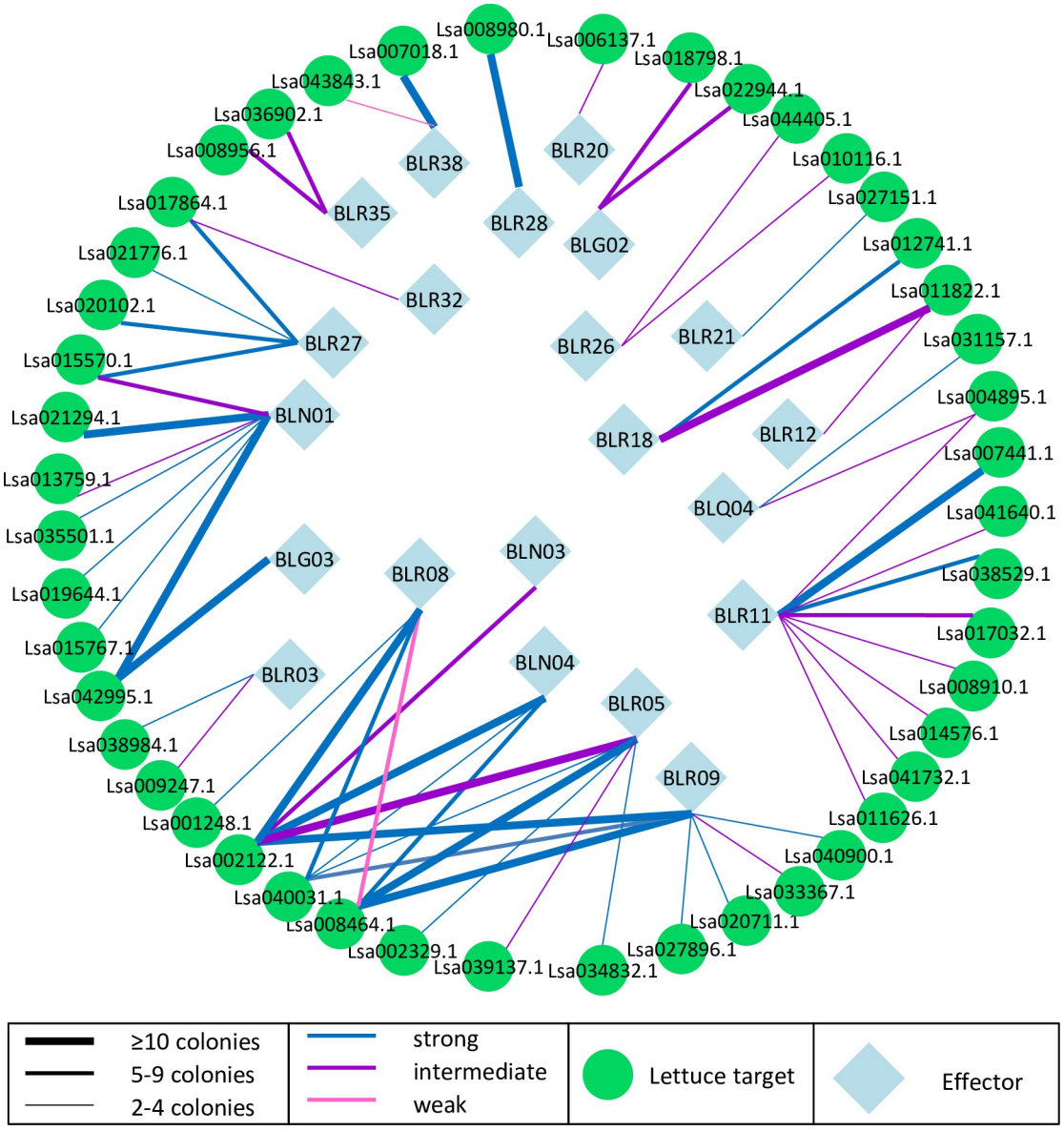

Fig 1. Yeast-two-hybrid identified *B. lactucae* effector–lettuce protein interactions. Interactions between *B. lactucae* effectors (blue diamonds) and lettuce proteins (green circles) are depicted according to the number of independent colonies representing a lettuce interactor and the level of reporter gene activation.

https://doi.org/10.1371/journal.pone.0226540.g001

observed free YFP on the immunoblots originates from degradation occurring during protein extraction.

Of the 15 effectors described here, four localized exclusively to the cytoplasm (BLN01, BLR11, BLR20, and BLR27) and two (BLG02 and BLR18) showed both cytoplasmic and nuclear localization (Fig 4 and Table 3). In contrast, YFP-BLR28 and BLR38-YFP localized solely to the nucleus (also see [12]), as expected because they are predicted to have nuclear localization signals (NLSs) (S2 Table). A predicted C-terminal transmembrane domain is present in six effectors (Table 1 and S3 Table) and these were thus expected to localize to membranes. We were unable to detect BLN03 and BLN04 C- or N-terminal fusion proteins, resulting in localizations for 13 of the 15 tested effectors (Fig 4). YFP-BLR05 and YFP-BLR09

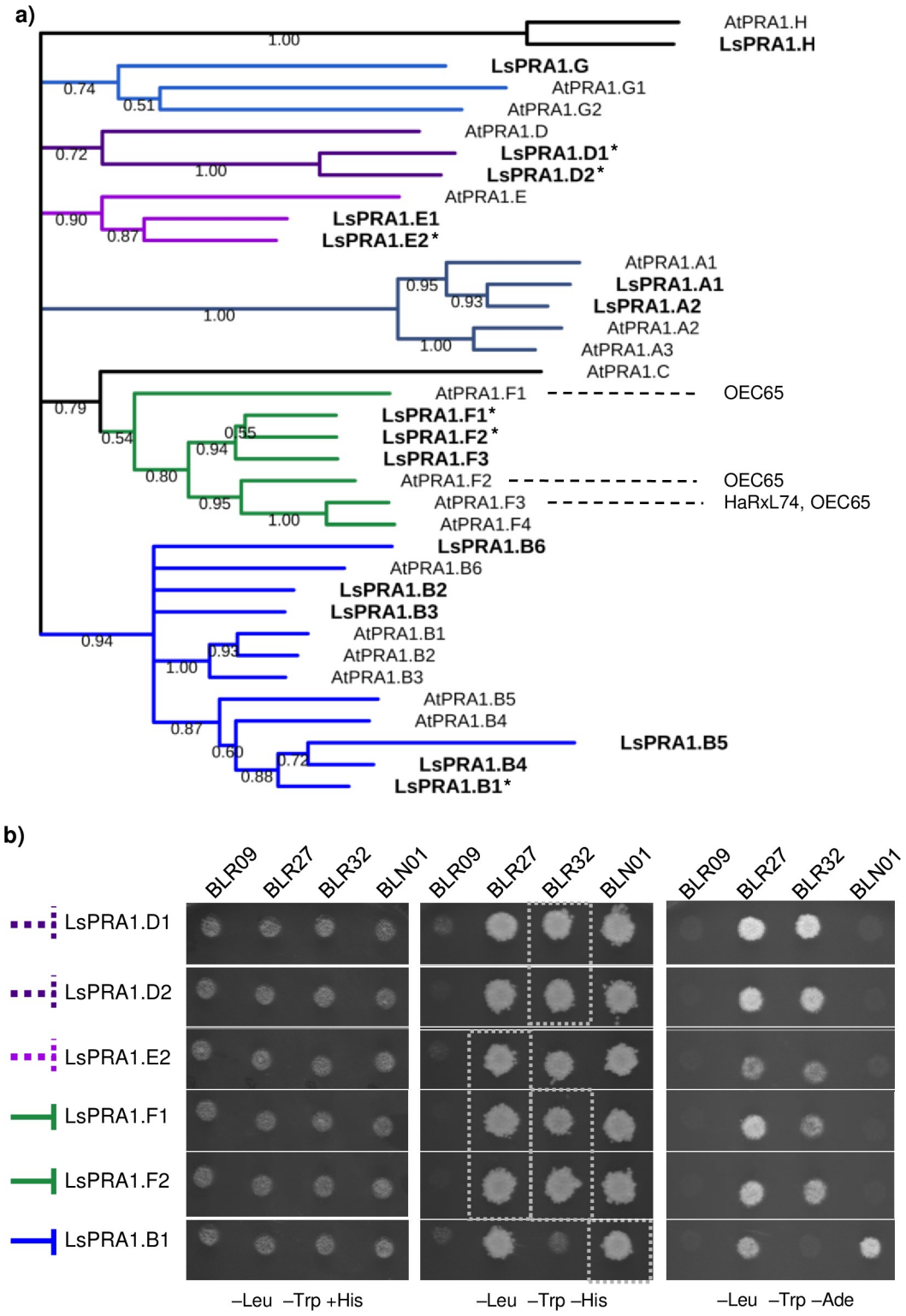

Fig 2. Three *B. lactucae* effectors interact with multiple PRA1 family members. A) Phylogenetic clustering of 17 lettuce and 19 Arabidopsis PRA1 domain-containing proteins. Sequences were aligned using Clustal Omega. Tree construction was performed in MEGA 7.0 using Neighbor-Joining with partial deletion of sites with less than 95% coverage. Nodes with less than 50% confidence score based on 1000 bootstrap replicates were removed. The previously reported interactions between *Hyaloperonospora arabidopsidis* and *Golovinomyces orontii* effectors HaRxL74 and OEC65 and Arabidopsis PRA1 proteins have been added. An asterisk (*) behind the protein indicates it was included in the targeted Y2H. B) A targeted Y2H was

performed with yeast isolated prey plasmids containing gene fragments of six PRA1 family members using *B. lactucae* effectors BLR27, BLR32 and BLN01 as bait. BLR09 was included as a negative control bait. Dashed line indicates the PRA1 protein did not pass all Y2H selection criteria, a continuous line indicates the PRA1 proteins passed the Y2H selection criteria. Left: permissive plate containing histidine. Middle: moderately selective plate lacking histidine. Right: strongly selective plate lacking adenine. The positions of previously identified interactions in the library screens are indicated with grey blocks for selection on SC medium lacking histidine.

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predominantly labeled the endoplasmic reticulum (S1 Fig), whereas YFP-BLR08 was associated with ring-like structures of various sizes in line with our previously reported findings [32]. YFP-BLR12 appeared as punctate structures. Upon prolonged overexpression, YFP-BLR12 also labeled the endoplasmic reticulum suggesting that the punctate structures represent components of the secretory pathway, that, however, requires confirmation using appropriate markers. Interestingly, BLG03 that is recognized in *Dm2* lettuce lines [11], does not contain predicted transmembrane domains, but YFP-BLG03 was associated with the plasma membrane (S1 Fig) similarly to BLN06-YFP and BLR40-YFP [12]. It is possible that post-translational modifications (S4 Table) contribute to observed membrane localizations.

Subsequently, the localization of tagged lettuce proteins was analyzed. Seven of the twelve tested proteins localized to the cytoplasm and/or nucleus (Fig 5 and Table 3). LsFLX-like2 and Lsa021294.1 localization was restricted to small, punctate structures in the cytoplasm. In line with the absence of signal peptide or transmembrane domain in LsFLX-like2 and Lsa021294.1, no ER localization was observed. The structures are thus more likely to present cytoplasmic protein complexes than components of the secretory pathway. However, for now, their origin remains undetermined. We were unable to detect C- or N-terminal LsRTNLB05 fusion proteins, resulting in localizations for 11 of the 12 interactors from lettuce (Fig 5). LsNAC069 and Lsa008464.1 labelled the endoplasmic reticulum (S2 Fig).

Upon co-expression of the effectors with lettuce proteins, complete or partial colocalization was observed for 10 out of 13 combinations, entailing 11 effectors and 10 lettuce interactors

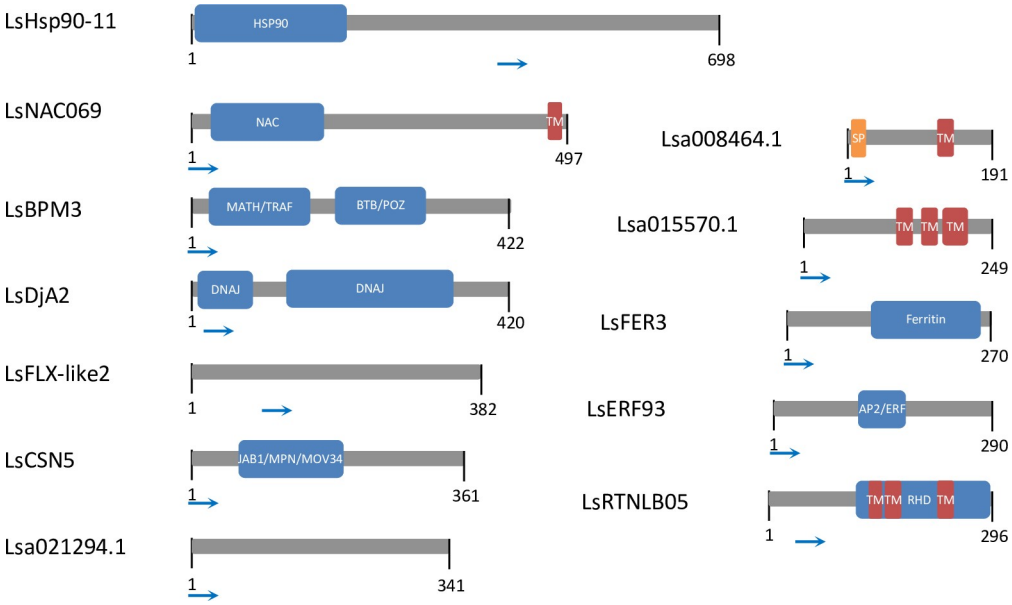

Fig 3. Graphical representation of Y2H-identified effector interactors. The start position of the longest identified cDNA fragment is indicated with a blue arrow for each ORF. Domains were predicted using InterProScan. TM = transmembrane domain, SP = signal peptide.

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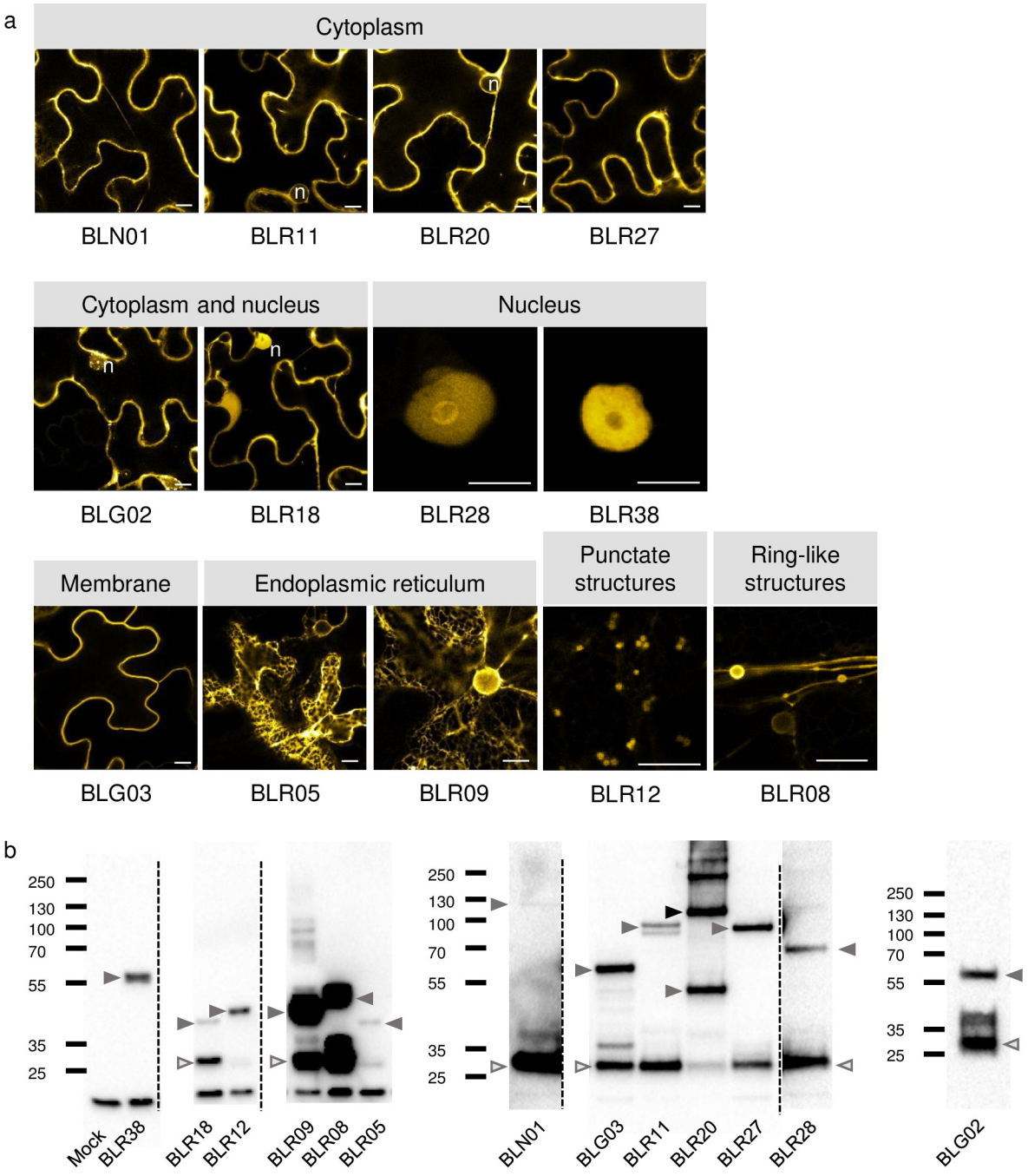

Fig 4. Subcellular localization and stability of 13 *B. lactucae* effectors in *N. benthamiana*. Effectors were fused N-terminally to YFP at the predicted signal peptide cleavage site, with the exception of BLR38 that was cloned as a C-terminal fusion protein, and transiently expressed in *N. benthamiana* using *Agrobacterium*. a) Images were taken 2–3 dpi. Bars = 10 μm. n = nucleus b) Immunoblots of the fusion proteins indicating their size and stability. Most effector fusion proteins are predominantly present in monomeric form with only a single band at the expected height of the fusion protein and occasionally lower bands that may represent cleavage products. YFP-BLR20 forms an exception as besides a band corresponding to the monomer, equally intense bands are present at twice and four times the weight of YFP-BLR20 indicating that it forms (stable) dimers and multimers under the denaturing conditions used. Grey arrows indicate the monomeric size of the fusion proteins, black arrows indicate multimers and open arrows indicate free YFP. Data on BLR05 and BLR09 have been published earlier [32].

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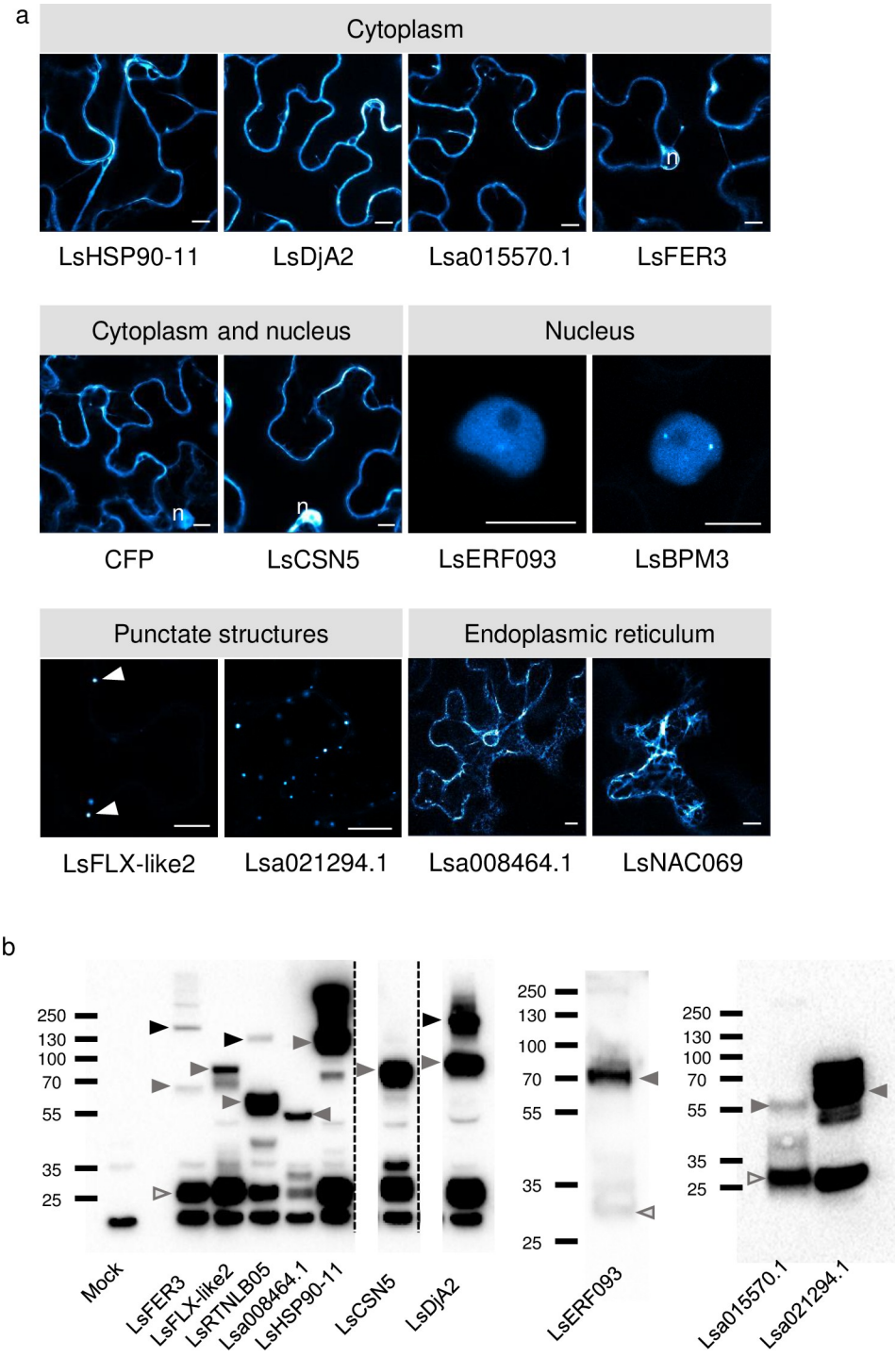

Fig 5. Subcellular localization and stability of 11 effector interactors in *N. benthamiana*. Lettuce proteins were fused N-terminally to CFP and transiently expressed in *N. benthamiana* using *Agrobacterium*. a) Images were taken 2–3 dpi. Bars = 10 μm. n = nucleus, arrowheads indicate the position of punctate structures. b) Immunoblots of the fusion proteins indicating their size and stability. Grey arrows indicate the monomeric size of the fusion proteins, black arrows indicate multimers and open arrows indicate free YFP.

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Table 3. Subcellular localization of FP-fusions of 11 effectors and 10 corresponding lettuce proteins, expressed individually or together to determine possible co- or re-localization.

Single expression of *B. lactucae* effector		**Single expression of lettuce protein**		**Co-expression of effector and lettuce interactor**		
Effector	*Subcellular localization*	*Lettuce protein*	*Subcellular localization*	*Subcellular localization effector*	*Subcellular localization lettuce protein*	*Colocalization*
BLG02	Cytoplasm and occasionally nucleus	LsBPM3	Nucleus	Cytoplasm and nucleus	Nucleus	Yes
BLG03	Plasma membrane	LsFER3*	Cytoplasm	Plasma membrane	Cytoplasm	No
BLN01	Cytoplasm			Cytoplasm	Cytoplasm	Yes
		Lsa015570.1*	Cytoplasm	Cytoplasm	Cytoplasm	Yes
		Lsa021294.1	Punctate structures	Cytoplasm and punctate structures	Punctate structures	No
BLR05	Endoplasmic reticulum	Lsa008464.1	Endoplasmic reticulum	Endoplasmic reticulum	Endoplasmic reticulum	Yes
BLR11	Cytoplasm	LsHSP90-11	Cytoplasm	Cytoplasm	Cytoplasm	Yes
BLR12	Punctate structures	LsCSN5*	Cytoplasm and nucleus	Punctate structures	Cytoplasm and nucleus	No
BLR18	Cytoplasm and nucleus			Cytoplasm and nucleus	Cytoplasm and nucleus	Yes
BLR20	Cytoplasm	LsERF093	Nucleus	Cytoplasm and nucleus	Nucleus	Yes
BLR27	Cytoplasm	Lsa015570.1*	Cytoplasm	Cytoplasm	Cytoplasm	Yes
BLR28	Nucleus	LsDjA2	Cytoplasm and occasionally nucleus	Nucleus	Cytoplasm and nucleus	Yes
BLR38	Nucleus	LsFLX-like2	Punctate structures	Nucleus	Nucleus	Yes

*Three lettuce proteins were tested for their interaction with two different effectors and therefore occur twice in the table.

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(Fig 6 and Table 3). As expected, the cytoplasmic localized effectors YFP-BLR11, YFP-BLR27 and YFP-BLN01 colocalized upon co-expression with their cytoplasmic interactors CFP-LsHSP90-11, CFP-Lsa015570.1 and CFP-LsFER3. Interestingly, in several cases co-expression induced a relocalization, specifically, to the nucleus.

To appreciate the relocalization phenomenon we present the localization of singly expressed proteins besides the co-expressed proteins for a number of interactions (Fig 7). Relocalization to the nucleus of effector BLG02 (mainly cytoplasmic, although in some cells also weakly nuclear) occurred in the presence of the nuclear-localized protein CFP-LsBPM3 (Fig 7). The cytoplasmic effector YFP-BLR20 relocalized to the nucleus (Fig 7) upon co-expression of nuclear localized CFP-LsERF093 and the intensity of the YFP nuclear signal was dependent on CFP-LsERF093 signal intensity.

In other cases, effector expression led to relocalization of the interactor. Effector BLR38-YFP induced a relocalization of its interactor CFP-LsFLX-like2 from punctate cytoplasmic structures to the nucleus (Fig 7). Also, nuclear-localized effector YFP-BLR28 induced relocalization of CFP-LsDjA2 to the nucleus (Fig 7). LsDja2 was predominantly cytoplasmic and in some cells nucleocytoplasmic upon single expression, but co-expression with BLR28 induced a shift towards nucleocytoplasmic localization. In summary, ten of the thirteen effector-lettuce protein pairs colocalized in *N. benthamiana*. Furthermore, relocalization occurred upon expression of four effector-lettuce protein pairs providing evidence that the Y2H-identified interactions also occur *in planta*.

Discussion

Features of the *B. lactucae* effectors

RxLR effectors are restricted to oomycetes belonging to the clade of the Peronosporales. In each species a number of these effectors contain structurally conserved WY domains that

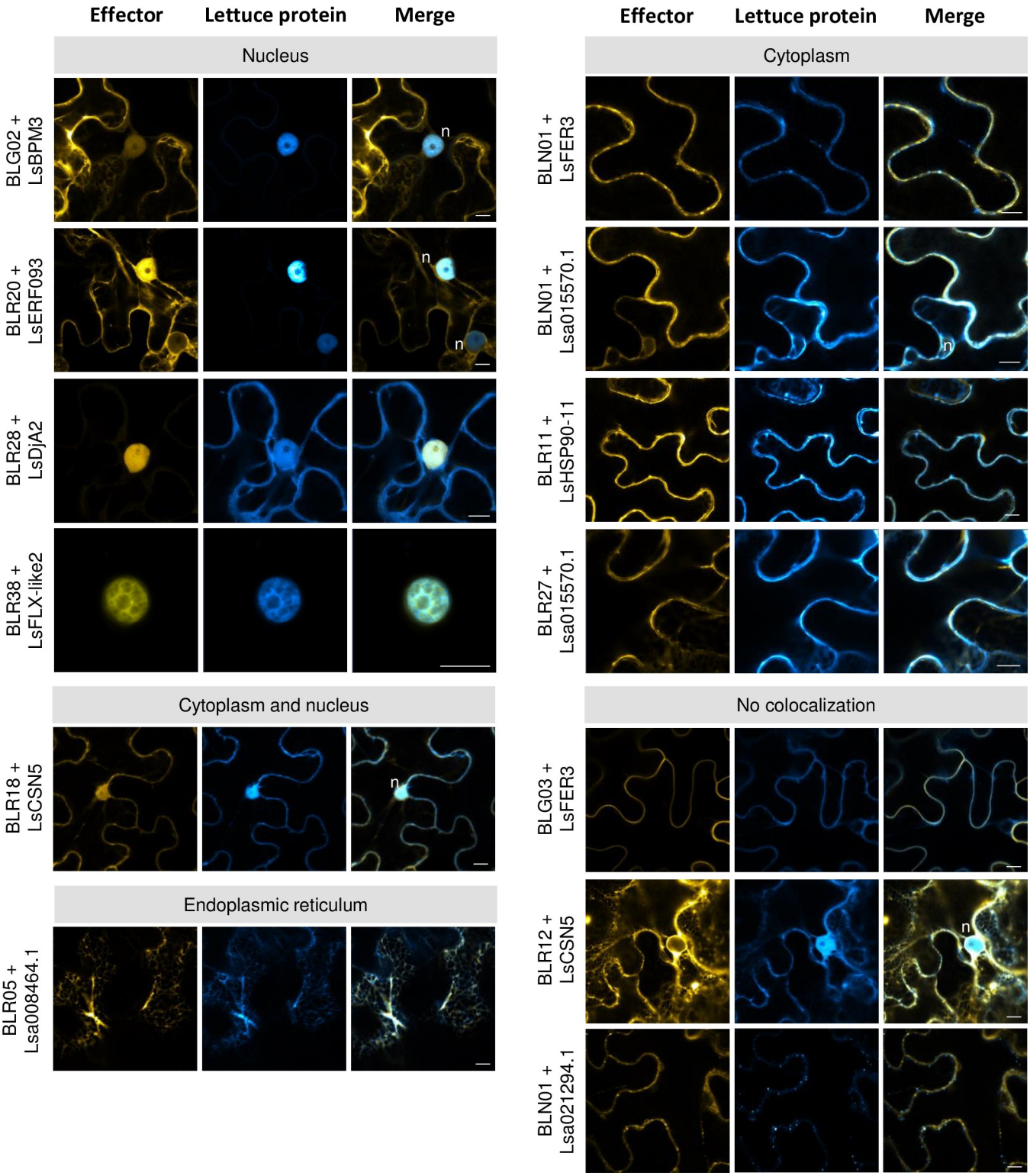

Fig 6. Colocalization of *B. lactucae* effectors and their interactors in *N. benthamiana*. *B. lactucae* effectors and lettuce proteins were transiently co-expressed in *N. benthamiana* using *Agrobacterium*. Images were taken 2–3 dpi. Bars = 10 μm. n = nucleus.

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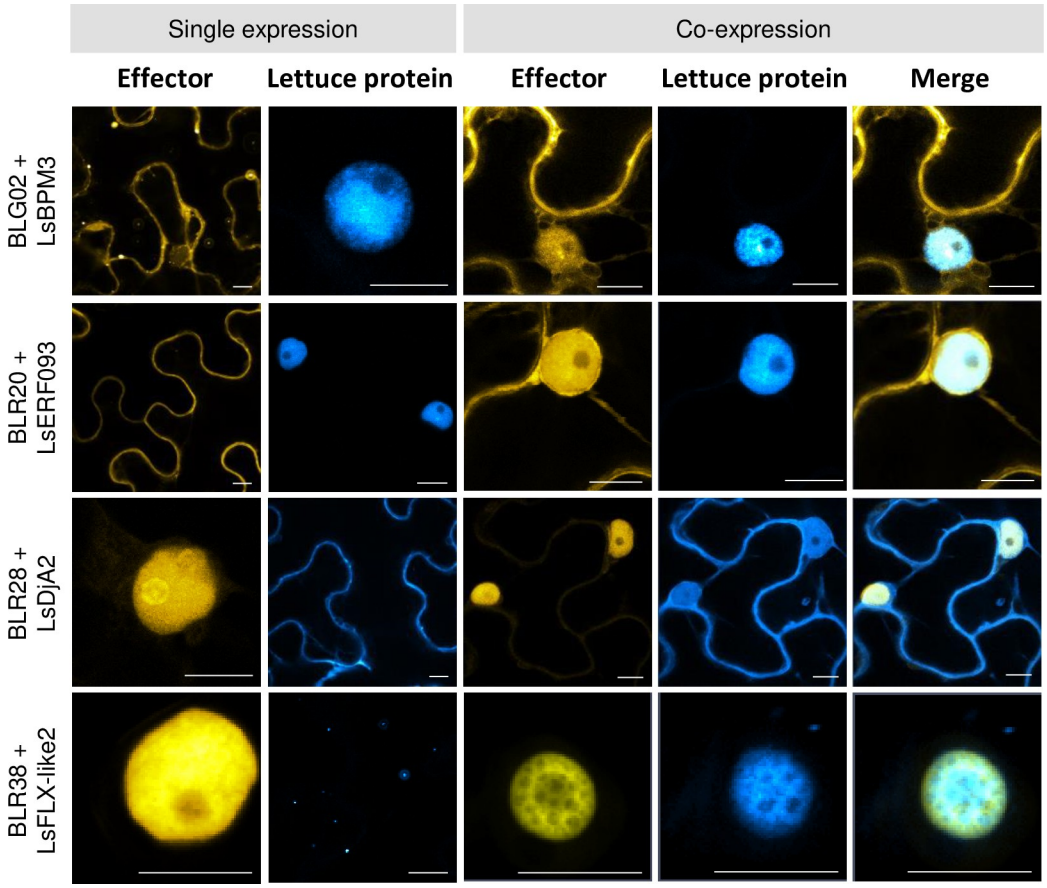

Fig 7. Relocalization of *B. lactucae* effectors or their interactors in *N. benthamiana*. *B. lactucae* effectors and lettuce proteins were transiently expressed separately or co-expressed in *N. benthamiana* using *Agrobacterium*. Images were taken 2–3 dpi. Bars = 10 μm.

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adopt an α-helical fold supported by W/Y/L sequence motifs [23,30]. The WY-domain is present in 44% of the Phytophthora RxLR effectors, but is less abundant in downy mildews: 18% of the *Plasmopara halstedii* [29] and 26% of the *H. arabidopsidis* [23] RxLR effectors contain WY domains. Our analysis revealed that from the set of 46 *B. lactucae* effectors originating from isolate Bl:24 seven (15%) effectors have predicted WY domains. These seven effectors were also identified in isolate SF5 [33]. The differences in WY-domain abundance may reflect a bias in the WY-domain algorithm towards *Phytophthora* sequences; other downy mildew effectors may contain a, so far, unrecognized conserved fold.

Yeast two-hybrid interactions between *B. lactucae* effectors and lettuce proteins

The cDNA library-based Y2H screen resulted in lettuce interactors for 21 (46% of screened) effectors. These effectors interacted on average with three lettuce proteins. The vast majority (83%) of identified lettuce proteins interacted only with a single *B. lactucae* effector. Previously published screenings with *C. elegans* and human fragment libraries resulted in interactors for 37% and 31% of bait proteins respectively corresponding to an average of 2.2 and 2 interactors per bait [34,35]. An average of 3.4 interactors per effector was obtained in screens with effectors from *G. orontii*, *H. arabidopsidis* and *P. syringae* against a library of ~8000 immune-

related full-length Arabidopsis proteins [8,9]. The number of obtained interactors in our screen is thus comparable to other published Y2H screens.

Based on the identification of Arabidopsis proteins that interacted with effectors from three pathogens [9], it has been proposed that effectors converge on conserved host proteins. This proposition fits with insights into the mechanisms by which independent bacterial type III effectors converge on immune-related proteins such as MAPK proteins [36], SERK3/BAK1 [37–41] and RIN4 [42]. We were interested to determine if those proteins identified as major hubs, i.e. interacting with effectors from multiple pathogens, would also emerge in our screens with *B. lactucae* effectors.

The list of Arabidopsis hubs was dominated by TCP transcription factors [9]. Specifically, TCP13, TCP14 and TCP15 interacted with effectors originating from all three pathogens, and TCP19 and TCP21 interacted with effectors from at least one pathogen [9]. TCPs operate as transcriptional activators or repressors in plant growth and development [43,44]. Lettuce TCP family members were also detected as interactors of *B. lactucae* effectors. However, the effector-TCP interactions weakly activated the *HIS3* reporter, were frequently observed as single colonies in Y2H screens and TCPs showed autoactivation in the Y2H system. Weßling and colleagues also classified Arabidopsis CSN5A as a hub due to its interaction with 12 *P. syringae* effectors, 11 *H. arabidopsidis* effectors and 9 *G. orontii* effectors in Y2H screens [8,9]. In our library screens, lettuce CSN5 was identified with two *B. lactucae* effectors, but also showed weak activation of the *HIS3* reporter in the presence of an empty bait vector. Interaction between CSN5 and the GAL4 DNA binding domain was previously reported using multiple GAL4-DBD based vectors and yeast strains [45–47]. Rejecting CSN5 as false positive because of stronger reporter gene activation in the presence of *B. lactucae* effectors than empty vector is only partially defendable. Effectors may have unforeseen additive effects on reporter gene activation but these effects could be interaction independent. Thus, though some of the previously reported hubs may represent host proteins that play a prominent role in disease susceptibility, others may be false positives.

Several *B. lactucae* effectors localize to membranes *in planta*

We examined the localization of a subset of 15 *B. lactucae* effectors and assessed their stability using immunoblot. Analysis of the YFP-BLR20 fusion protein on an SDS-PAGE gel under denaturing conditions revealed multiple bands corresponding to monomeric, dimeric and multimeric forms of this effector. It is possible that a multimer represents the biologically active form of BLR20 as multimerization of two *P. infestans* effectors, PexRD2 and PiSF3, was shown to be required for interaction with their targets, a host MAP kinase and U-Box-kinase protein respectively [23,48,49]. The multimerization of BLR20 is possibly driven by the presence of predicted WY-domains that have been proposed to support oligomerization [23]. This was previously shown for PiSF3 by mutation of two residues facing each other across the α-helices of PiSF3 that disrupted the formation of oligomers [48]. Interestingly, multimerization of PexRD2 and PiSF3 was not demonstrated under denaturing conditions. Some transmembrane domains or transmembrane domain containing proteins give two bands corresponding to the monomer and multimer under denaturing conditions [50]. More research is required to determine if non-covalent interactions between hydrophobic interfaces of WY domains cause the observed multimerization or if other factors play a role.

B. lactucae effectors BLG03, BLN06 and BLR40, which are recognized and trigger HR in specific lettuce lines, localized to the plasma membrane [11,12]. Plant membranes were also targeted by 26% of 49 tested *H. arabidopsidis* effectors [51] and 12% of 76 tested *Plasmopara viticola* effectors [52]. Coiled-coil domains [53] and post-translational modifications such as

N-myristoylation, could contribute to anchoring of effectors, lacking transmembrane domains, to the plasma membrane [51,54,55]. Six effectors—BLN03, BLN04, BLR05, BLR08, BLR09 and BLR12 –were predicted to contain a single C-terminal transmembrane domain based on TMHMM analysis. Effectors BLR05, BLR08, BLR09 and BLR12 were indeed found to be associated with the endomembrane system: YFP-BLR05, YFP-BLR09 [32] and YFP-BLR12 strongly labelled the endoplasmic reticulum, whereas BLR08 was associated with ring-like structures of varying sizes. Recently, it has been shown that BLR05 and BLR09 co-localized with their interactor LsNAC069 at the endoplasmic reticulum [32]. The *B*. *lactucae* effectors were cloned without their signal peptide, which is required for co-translational ER targeting of transmembrane proteins by the signal recognition particle [56]. Instead membrane integration of tail-anchored proteins occurs post-translationally and is dependent on a C-terminal transmembrane domain that functions as a targeting signal. Some proteins with a moderately hydrophobic TM domain can insert unassisted in the ER, others with a highly hydrophobic TM domain require assistance. In yeast, the TM domain of tail- anchored proteins is recognized by components of the GET system including Get3 (TRC40 in mammals), delivered to the ER-resident Get1/Get2 receptor complex and inserted into the membrane [57–60]. Clearly, the ER localization of YFP-BLR05, YFP-BLR09 and YFP-BLR12 proves that these effectors associate with the membrane post-translationally. This immediately prompts the question how membrane-associated effectors are delivered to host cells. A role for extracellular vesicles in the delivery of soluble effectors has been hypothesized [61] and vesicular transport would provide a suitable vector for membrane-associated effectors as well. The C-terminal transmembrane domain of YFP-BLN03 and YFP-BLN04 may not have been sufficient for posttranslational insertion providing a possible explanation for the lack of fluorescence of these fusion proteins.

Effector BLG03 targeted the plasma membrane despite lacking a putative transmembrane domain. Post-translational modifications resulting in protein lipidation such as prenylation, N-myristoylation or palmitoylation can affect membrane-protein interactions [62]. Putative N-myristoylation sites were implicated in the membrane targeting of multiple bacterial type III effectors [55,63]. Alternatively, phospholipid conjugation [62] or phospholipid binding may contribute to membrane association. Multiple studies have explored the role of the N- terminal RXLR motif and C-terminal residues in phospholipid binding [64]. Though the relevance of phospholipid binding for effector uptake remains unclear, perhaps a role in effector localization *in planta* could be considered.

Co-expression of effectors and interactors induces protein relocalization to the nucleus

Studies on *H*. *arabidopsidis* and *P*. *viticola* RXLR effector localization revealed a preference for nuclear (including nucleocytoplasmic) localization (66% and 83% respectively) [51,52]. In our research, only two effectors–BLR28 and BLR38 –were strictly nuclear localized in *N*. *benthamiana*. Strikingly, *B*. *lactucae* RXLR effector BLR38 that is recognized in *L*. *serriola* LS102, induced a relocalization of its interactor LsFLX-like2 to the nucleus. LsFLX-like2 shows homology to the Arabidopsis FLOWERING LOCUS C EXPRESSOR (FLX) protein family of which two members are involved in flowering time control in Arabidopsis [65]. The punctate structures of CFP-LsFLX-like2 resemble the structures formed by cytoplasmic FLX homodimers in Arabidopsis [66]. Heterodimers of FLX with FRIGIDA or FRIGIDA ESSENTIAL 1 were restricted to the nucleus [66], suggesting that the observed relocalization of CFP-LsFLX-like2 upon co-expression with BLR38-YFP may reflect a different interaction state. The identification of LsFLX-like2 in a screen for *B*. *lactucae* effectors may hint towards a role of FLX-like

family members in the integration of environmental signals in flowering time regulation. Bacterial, fungal and downy mildew infection induce early flowering of Arabidopsis plants [67,68] although the molecular mechanism by which biotic stress is integrated in flowering time regulation remains poorly understood. Thus, it would be interesting to explore if *B. lactucae* infection affects regulation of flowering time in lettuce. BLR28 also induced relocalization of its interactor, DnaJ protein LsDjA2, to the nucleus. DnaJ proteins function as co-chaperones to 70 kDa heat shock proteins to aid in protein folding, relocalization and degradation [69].

Furthermore, two predominantly cytoplasmic effectors, BLG02 and BLR20, displayed nucleocytoplasmic localization when co-expressed with their interactors, LsBPM3 and LsERF093. LsERF093 contains an AP2/ERF domain that is typically found in members of the AP2/ERF transcription factor family. These proteins are known to be involved in responses to biotic and abiotic stress in Arabidopsis, tomato and rice [70,71]. LsBPM3 contains a BTB/POZ (broad complex, tram track, bric-a-brac/POX virus and zinc finger) domain that is also found in 80 Arabidopsis proteins and is known to mediate protein-protein interactions. Specifically, the BTB/POZ domain acts as a substrate-specific adaptor for Cullin-RING E3 ubiquitin Ligases (CRLs) that ubiquitinate proteins targeted for degradation by the proteasome. Recruitment of substrates may occur via a Meprin and TRAF homology (MATH) domain [72,73]. The Arabidopsis MATH-BTB/POZ protein BPM3 localizes to the nucleus and promotes degradation of transcription factor ATHB6 that negatively regulates abscisic acid signaling [74]. Furthermore, BPMs interact with APETALA 2/ethylene-responsive element binding factor (AP2/ERF) transcription factors [75].

The relocalization events that we observed, have two important implications. First, they suggest that physical interactions between RXLR effectors and interactors occur *in planta*, which may affect the activity of the interactor and consequently, disease development. This principle was recently demonstrated for *P. sojae* effector Avh52 that recruits a cytoplasmic host transacetylase, GmTAP1, into the nucleus. Nuclear-localized GmTAP1 is able to transacetylase histones, which enhances susceptibility of *N. benthamiana* to *P. capsici* [76]. Also, Arabidopsis protein RD19 that was discovered as interactor of *Ralstonia solanacearum* type III effector Pop2 in a Y2H screen, relocalized to the nucleus in the presence of the effector and physically associated with Pop2. The effector target, RD19, was required for the resistance protein RRS1-R mediated immune responses [77] Thus, relocalization events are often associated with physical interactions between effector and interactor, and effector-target interactions can affect disease susceptibility. Functional analysis of the lettuce interactors is required to provide further insights into the mechanisms underlying *B. lactucae* susceptibility.

The second implication is that screening by Y2H is a useful method to identify potential host targets. However, the biological relevance of the observed interactions needs to be demonstrated in follow-up experiments and there is no universal rule (yet) for selecting the most promising candidates. We investigated LsFLX-like2/ BLR38 and LsDjA2/ BLR28 because they belonged to the top ten ranked interactions in the Y2H screen. In contrast, BLG02/ LsBPM3 and BLR20 / LsERF093 were selected based on literature demonstrating a role for the target candidate protein families in immune responses. It should furthermore be stressed that neither of these four interactors function as hub in our Y2H screen: all of them interacted with a sole effector. Conversely, we have previously demonstrated that small networks identified in Y2H screens can be valuable i.e. LsNAC069 interacted with four—BLN04, BLR05, BLR08 and BLR09—effectors in Y2H assays and was demonstrated to affect plant responses to both biotic and abiotic stress [32]. To conclude, our Y2H screens have uncovered multiple potential effector targets whose precise function we are just beginning to unravel.

Supporting information

S1 Table. Primers used in this study.
(DOCX)

S2 Table. Prediction of importin-α-dependent nuclear localization signals.
(DOCX)

S3 Table. Position of transmembrane domains in effectors using TOPCONS.
(DOCX)

S4 Table. Putative post-translational lipid modifications in membrane-associated effectors.
(DOCX)

S1 File. Supplemental material and methods.
(DOC)

S2 File. New gene names for lettuce gene models.
(XLSX)

S1 Fig. Co-localization of effectors with markers in *N. benthamiana*. A) YFP-BLR05 and YFP-BLR09 co-localize with an CFP-tagged ER lumenal marker. B) YFP-BLG03 localizes to the plant plasma membrane. Leaf sections were incubated in propidium iodide (PI) to stain the cell wall. Bars = 10 μm.
(TIF)

S2 Fig. LsNAC069 and Lsa008464.1 co-localize with an ER lumenal marker in *N. benthamiana*. Bars = 10 μm.
(TIF)

S3 Fig. Phylogenetic tree of lettuce BTB/POZ domain containing proteins. The Y2H identified target, LsBPM3, forms a distinct clade with four other lettuce BTB/POZ domain containing proteins and six Arabidopsis BPM proteins. AtBPM1-AtBPM6 contain in addition to a BTB/POZ domain also a MATH domain. Protein sequences were aligned using Clustal Omega, a Neighbor-Joining phylogenetic tree was constructed using MEGA7 and the tree was visualized using iTOL software.
(TIF)

S4 Fig. Phylogenetic tree of lettuce and Arabidopsis FLX(-like) proteins. Protein sequences were aligned using Clustal Omega, a Neighbor-Joining phylogenetic tree was constructed using MEGA7 and the tree was visualized using iTOL software.
(TIF)

S1 Raw images. Raw images western blots.
(PDF)

Acknowledgments

We would like to thank our collaborators for their support: Wageningen University (Wageningen, The Netherlands), Bayer, now BASF (Nunhem, The Netherlands), Enza Zaden (Enkhuizen, The Netherlands), Syngenta (Enkhuizen, The Netherlands), RijkZwaan (De Lier, The Netherlands) and Vilmorin & Cie (Lab Ménitré, France).

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