Expression of the immunoreactive buckwheat major allergenic storage protein in *Lactococcus lactis*

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Abstract

Proteins from buckwheat (*Fagopyrum esculentum*) are strong allergens that can cause serious symptoms, including anaphylaxis, in patients with hypersensitivity. In this study, we successfully developed a modified lactic acid bacterial vector (pNSH) and a recombinant strain of *Lactococcus lactis* NZ9000 (NZ9000) that produced a major allergenic storage protein of buckwheat, Fagag1 (61.2 kDa, GenBank accession number AF152003), with or without a green fluorescence protein (GFP) tag. GFP fluorescence allows for rapid, simple, and accurate measurement of target protein expression by microscopy or fluorimetry. We describe a convenient method for production of rGFP-Fagag1 fusion and rFagag1 proteins with a good yield in an advantageous probiotic host. We found that *in vitro* treatment of splenocytes isolated from buckwheat crude protein-immunized mice with rFagag1 increased expression of allergic inflammation cytokines such as IL-4, IL-13 and IL-17F. Because it was less antigenic, rGFP-Fagag1 protein from NZ9000 might be of limited use; however, rFagag1 from NZ9000 evoked a robust response as measured by induction of IL-4 and IL-17F expression levels. The observed allergic activity is indicative of a Th2-cell mediated immune response and is similar to the effects induced by exposure to buckwheat crude protein. Our results suggest that expression of rFagag1 in NZ9000 may facilitate *in vivo* applications of this system aimed at improving the specificity of immunological responses to buckwheat allergens.

Keywords; Buckwheat, *Fagopyrum esculentum*, allergen, Fagag1, *Lactococcus lactis,* Th2

Introduction

Buckwheat (*Fagopyrum esculentum*) belongs to the Polygonaceae family of plants and its seeds can be used as food material. Buckwheat is a pseudocereal; that is, buckwheat is a eudicot with seed qualities and uses similar to those of monocot cereals (family Poaceae). Buckwheat can be cultivated under less favorable conditions than rice, including at high altitudes, and it is sometimes used as a substitute for common cereals. Buckwheat is consumed around the world as a health food (Sedej et al. 2012). In Japan, buckwheat is called “soba” and is consumed mainly in the form of noodles and dumplings. In Western countries, buckwheat is also used in batter for cakes, pancakes, or crepes, and there is growing interest in buckwheat as a health food and as a substitute for wheat flour for gluten-allergic persons (Panda et al. 2010).

Buckwheat is considered a true food allergen, also classified as a class I food allergen, comparable to the major milk allergen (Ruiter et al. 2006), the fish allergen parvalbumin (Swoboda et al. 2002), major peanut allergens (Palmer K and Burks 2006), and the major shrimp allergen (Reese et al. 2005). Indeed, in affected people, buckwheat consumption can induce severe and even life-threatening anaphylactic reactions. To date, many cases of buckwheat allergies have been reported (De Maat-Bleeker and Stapel 1998; Yoshioka et al. 2004). Buckwheat allergens induce immunoglobulin E (IgE)-mediated allergies in the gastrointestinal system, skin, and respiratory track, and can evoke severe reactions, such as anaphylaxis (Varga et al. 2011; Heffler et al. 2011). A system for the delivery of buckwheat allergen via mucosal immunization has not previously been developed. However, we speculate that such a system would aid in the study and modulation of T helper type 2 (Th2) cell immune responses.

In this study, we focused on a major allergenic storage protein from buckwheat, Fagag1. A Fagag1 sequence was identified based on NCBI Genbank accession number AF152003. This Fagag1 sequence has high identity to two cDNA clones isolated from a buckwheat cDNA library of developing seeds (defined as “buckwheat mRNA for legumin-like protein, complete cds” at NCBI; Accession numbers D87980 and D87982). Minor differences in sequence length and deduced amino acid sequences among these sequences may be due to isolation of different isoforms from different sources, or could be variety dependent.

Current evidence suggests that probiotic bacteria can play a beneficial role in the prevention or treatment of Th2-biased allergic responses (Kitazawa et al. 2008; Iemoli et al. 2012; Ou et al. 2011). *Lactococcus (Lc.) lactis* is a highly efficient probiotic microorganism with a wide range of benefits for human health (Kitazawa et al. 2008). Experimental data and genomic analyses indicate that *Lc. lactis* naturally secretes only a few proteins (Poquet et al.1998), and a plasmid-free *Lc. lactis* strain does not produce the extra cytoplasmic protease PrtP (Gasson 1983). These features have drawn the attention of researchers to the potential use of *Lc. lactis* for the secretion of biotechnologically important proteins. The nisin-controlled gene expression (NICE) system has become a widely used tool for regulated gene expression in Gram-positive bacteria (Bryan et al. 2000), including lactic acid bacteria such as *Lc. lactis*. Use of the NICE system in lactic acid bacteria might provide a good approach for generating a novel topical antigen delivery vehicle for mucosal immunization (Mercenier et al. 2000), for example for buckwheat proteins. However, there are no previous reports of such a buckwheat protein expression system in lactic acid bacteria.

Here, we engineered a *Lc. lactis* NZ9000 (NZ9000) recombinant strain that produces GFP fused to Fagag1 (rGFP-Fagag1). The GFP fluorescence signal allows for rapid, simple, and accurate measurement of target protein expression. The aim of the present study was to design a high-level expression system for recombinant Fagag1 and a purification method based on GFP fluorescence that can be used for both small- and large-scale cultures. Development of such a system should facilitate investigation of the immunological activity of splenocytes isolated from mice immunized with buckwheat crude protein and subsequently treated with rFagag1 or rGFP-Fagag1.

Materials and methods

Bacterial strain and growth conditions

NZ9000 is a standard host strain for the NICE system (MoBiTec, Goettingen, Germany). NZ9000 is derived from *Lc. lactis* subsp. *cremoris* MG1363 (MG1363) and contains the regulatory genes *nisR* and *nisK* integrated into the *pepN* gene. NZ9000 was grown in M17 medium supplemented with 0.5% glucose at 30°C, and gene expression was induced with nisin, as described previously (Kleerebezem et al. 1997, Shigemori et al. 2012). Plasmid maintenance was ensured by growing recombinant NZ9000 strains in medium supplemented with 20 μg/mL chloramphenicol.

Construction of a recombinant NZ9000 strain

The pNSH vector is a modified form of the *Lc. lactis* expression vector pNZ8148 (MoBiTec) and contains the PnisA promoter and a terminator sequence upstream and downstream, respectively, of a multicloning site (MCS). A 6xHis tag and factor Xa recognition site are positioned between the PnisA promoter and MCS (Fig. 1a). The

GFP (accession number: AB758275, http://getentry.ddbj.nig.ac.jp/getentry/ddbj/AB758275?filetype=html), Fagag1(accession number: AB758276, http://getentry.ddbj.nig.ac.jp/getentry/ddbj/AB758276?filetype=html) and GFP-Fagag1 coding sequences were optimized for MG1363 codon usage to increase expression levels in NZ9000. The codon-optimized GFP and Fagag1 genes were synthesized by Operon Biotechnologies (Tokyo, Japan) and subcloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA). The resulting plasmid was digested with *BamH*I and *Hind*III to excise the GFP, Fagag1 or GFP-Fagag1 gene cassette, which was then cloned into pNSH vector. The resulting plasmid (pNSH-GFP, pNSH-Fagag1, or pNSH-GFP-Fagag1) contained the GFP, Fagag1, or GFP-Fagag1 gene cassette under control of the nisin-inducible PnisA promoter (Fig. 1b, c, d). The plasmids were introduced in NZ9000 by electroporation using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Inc., CA, USA) following the manufacturer's instructions. The resulting NZ9000 recombinant strains were named NZ9000-GFP, NZ9000-Fagag1 and NZ9000-GFP-Fagag1. NZ9000 was also electroporated with the empty plasmid pNSH to generate a NZ9000 vector control strain (NZ9000-VC).

Small-scale expression of recombinant protein

Fresh medium was inoculated 1:20 (v/v) with an overnight culture and incubated statically at 30°C. To induce expression of the recombinant genes, strains were grown to an OD_{600} of 0.4, followed by induction with 100 ng/mL nisin for 3 h. To evaluate recombinant protein expression in NZ9000, preparation of cell fractions was performed based on the method described by Loir et al., 1998. Briefly, 2 mL of NZ9000 cultures at

a given OD_{600} were harvested by 5 min centrifugation at 4°C and 3,000 x*g*. Next, the supernatant was removed; the cell pellet was washed once with 1 mL of TES (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 25% sucrose); and the pellet was then resuspended in 70 µL/OD_{600} = 1 of TES containing lysozyme (1 mg/mL). After 30 min of incubation at 37°C, cells (30 µL/ OD_{600} = 1) were lysed with 20% SDS. For electrophoretic separation, equal volumes of 2x sample buffer were added to each sample and the samples were then boiled for 5 min.

The boiled samples were separated by 10% SDS-PAGE and then transferred onto PVDF membranes. Western blotting was performed with primary antibodies (Abs) against the 6xHis tag (BioLegend, Inc., San Diego, CA, USA), followed by incubation with HRP-conjugated secondary Abs (Sigma, St. Louis, MO, USA). Signals were visualized by treating the membranes with TMB peroxidase substrate.

Large-scale expression and purification of recombinant protein

Optimal parameters for rFagag1 and rGFP-Fagag1 expression were assessed by growth curves (OD_{600}), external pH, and when appropriate, GFP fluorescence signal, following induction with various nisin concentrations and incubation for various times (hr). After induction with nisin, a cell pellet obtained by centrifugation was broken by grinding with aluminum oxide powder (3 g per 1 g of cells; Wako, Osaka, Japan) for 15 min, followed by suspension of the broken cells in a standard buffer containing a protease inhibitor cocktail (Sigma). Protein content was measured using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Fluorescence, reported in relative fluorescence units (RFU), was measured using a Fluoroskan Ascent FL

Microplate Fluorometer (Thermo Scientific, Tokyo, Japan) by excitation at 485 nm and detection of emission at 538 nm.

The rGFP, rFagag1 and rGFP-Fagag1 proteins were purified using a HisTrap HP column (1 mL, pre-charged with Ni^{2+}; GE Healthcare Japan, Tokyo, Japan) under native conditions, according to the manufacturer's instructions. The flow-through fractions were collected by washing the column with 20 mM imidazole phosphate buffer (pH 8.0). The absorbed factions were eluted using 31.25, 62.5, 125, 250, and 500 mM imidazole phosphate buffer (pH 8.0). After dialysis, the eluted fractions were freeze-dried and analyzed by SDS-PAGE and Western blotting.

Buckwheat crude protein-immunized mice

Pathogen-free female BALB/c mice (4 weeks of age) were purchased from Japan SLC (Shizuoka, Japan) and housed under temperature- and light-controlled conditions. Mice were fed a standard diet of Labo MR Breeder (Nihon Nosan Co., Kanagawa, Japan) and sterile water *ad libitum*. After a preliminary breeding period of 2 weeks, BALB/c mice (6 weeks of age, *n=3*) were intraperitoneally (i.p.) sensitized once weekly for 3 weeks with 100 μg of buckwheat (*Fagopyrum esculentum*) crude protein in lyophilized form (GREER Laboratories Inc., Lenoir, NC, USA) and alum (allergen/adjuvant ratio of 1/50). All experimental procedures were conducted in accordance with the Regulations for Animal Experimentation of Shinshu University.

Immunoreactivity assay

The levels of immunoreactivity of rFagag1 and rGFP-Fagag1 were assessed by real-time quantitative PCR (qPCR), which was used to detect cytokine mRNA

175 expression induced by rFagag1 or rGFP-Fagag1 in splenocytes isolated from buckwheat
176 crude protein-immunized mice. Splenocytes were prepared using standard methods
177 (Shimosato et al. 2010, Ito et al. 2012) and were then cultured in medium at a final
178 concentration of 2 x 10^6 cells per well (total volume, 1 mL per well). Splenocytes were
179 stimulated with 10 μg/mL purified rFagag1, rGFP-Fagag1 or various concentrations of
180 buckwheat crude protein as a control for 72 h, harvested, and then monitored for
181 cytokine mRNA expression by qPCR as previously described (Shimosato et al. 2009;
182 Shigemori et al. 2012). Fluorescent qPCR reactions were performed using SYBR
183 Premix Ex Taq (Takara Bio Inc.) and specific primers, with each reaction containing 5
184 ng of cDNA in a total volume of 25 μL. The β-actin and cytokine primers were
185 purchased from Takara Bio Inc. The PCR cycling conditions were 10 s at 95°C,
186 followed by 45 cycles of 5 s at 95°C, and 30 s at 60°C. As a control, poly $(A)^+$RNA
187 samples were used as templates to check for the presence of contaminating genomic
188 DNA. The sensitivity of the reaction and amplification of contaminant products, such as
189 the extension of self-annealed primers, were evaluated by amplification from various
190 serial dilutions of the cDNA template. For cross-sample comparison of results obtained
191 following various treatments, cytokine mRNA levels were first normalized to mRNA
192 levels obtained for β-actin. The results represent the means ± SD of three independent
193 experiments.
194
195 **Statistical analysis**
196 All results represent the average of three separate experiments. Statistical
197 analyses were performed using the *Bonferroni* multiple comparison tests for one-way

198 analysis of variance (ANOVA). Differences were considered significant when *P* values
199 were less than 0.05.
200
201 **Results**
202 **Construction of recombinant strains at a small scale**
203 We first modified the vector pNZ8148 to generate a vector for inducible
204 expression of tagged proteins, which we call pNSH. The pNSH vector contains an
205 N-terminal 6xHis tag, a factor Xa recognition site for cleavage of the 6xHis tag, and a
206 multiple cloning site (MCS) between a nisin-inducible promoter and a terminator
207 sequence (Fig. 1a). Full-length GFP, Fagag1, or both were isolated by restriction
208 enzyme digest and cloned into pNSH, resulting in pNSH-GFP, pNSH-Fagag1 and
209 pNSH-GFP-Fagag1 (Fig. 1 a-d). The plasmids were then electroporated into *Lc. lactis*
210 NZ9000. To confirm expression of the recombinant proteins in NZ9000, we next
211 analyzed protein fractions and whole cells from the transfected NZ9000 strains.
212 Following nisin-mediated induction, total cellular protein was separated by SDS-PAGE
213 and visualized with coomassie brilliant blue (CBB), then assayed for the presence of the
214 6xHis tag proteins via Western blotting (Fig. 2a,b). We monitored the recombinant
215 proteins by staining SDS-PAGE gels with CBB (Fig. 2a). However, the detection of
216 rGFP, rFagag1 and rGFP-Fagag1 were difficult in CBB-stained gels due to the weak
217 signal of recombinant proteins (Fig. 2a). In an empty vector control or in the absence of
218 nisin induction, recombinant proteins could not be detected in the NZ9000 strains (Fig.
219 2b). Following nisin-induced expression, however, bands corresponding to rGFP (30.6
220 kDa), rFagag1 (65.4 kDa) and rGFP-Fagag1 (91.7 kDa) were detected in cell fractions
221 from nisin-induced NZ9000-GFP, NZ9000-Fagag1, and NZ9000-GFP-Fagag1,

222 respectively (Fig. 2b). To confirm expression of rGFP, we next observed cells under
223 visible light (Fig. 3; light) and by confocal laser microscopy (Fig. 3; laser) following 3
224 hr of treatment with nisin. As shown in the merged images (Fig. 3; merge),
225 NZ9000-GFP and NZ9000-GFP-Fagag1 cells clearly express fluorescence recombinant
226 proteins (Fig. 3). Based on the results of Western blotting and confocal laser
227 microscopy, we concluded that we successfully induced expression of His-tagged and
228 GFP-fused Fagag1 recombinant proteins using NZ9000 transformed with the pNSH
229 vector containing the GFP, Fagag1 and GFP-Fagag1 gene cassette.
230
231 **Establishing optimal conditions for recombinant protein expression and**
232 **purification**
233 We used GFP fluorescence as an indicator of protein expression following
234 nisin-induced expression of GFP-Fagag1 in NZ9000-GFP-Fagag1. To optimize
235 induction, we assayed GFP intensity following treatment with various concentrations of
236 nisin for various times in large-scale cultures (*i.e.* 50 mL). When NZ9000-GFP-Fagag1
237 cells were stimulated with 62.5-500 ng/mL nisin, the resulting cell fractions significant
238 high levels of fluorescence compared with cell fractions from NZ9000-VC (Fig. 4a).
239 Thus, we determined that a nisin concentration of 100 ng/mL was optimal for inducing
240 expression of the recombinant protein, as indicated by the highest levels of fluorescence
241 observed at this concentration. The maximum detectable GFP fluorescence was
242 observed 1-3 hr after induction (Fig. 4b). Notably, in cell cultures treated with 100
243 ng/mL nisin, the external pH of the medium dropped from 6.5 to 5.0 (Fig. 4c) and
244 stationary phase was reached 4 hr after induction (Fig. 4d). We next used these optimal
245 conditions in very large-scale cultures (*i.e.* 3 L) in an effort to generate and isolate

246 significant quantities of the recombinant proteins. To do this, NZ9000-GFP,
247 NZ9000-Fagag1 and NZ9000-GFP-Fagag1 were induced by nisin in 3 L large-scale
248 culture, and rGFP, rFagag1 and rGFP-Fagag1 were purified using HisTrap HP column.
249 To confirm the purification of each recombinant protein, we analyzed the crude lysate
250 before purification, column flow-through and eluted fractions by SDS-PAGE and
251 Western blotting with anti-6xHis-tag Ab (Fig. 5a,b,c). A corresponding band with rGFP
252 (Fig. 5a), rFagag1 (Fig. 5b) or rGFP-Fagag1 (Fig. 5c) was observed in each eluted
253 fraction. The fraction eluted with 62.5 mM imidazole in particular was enriched for the
254 recombinant proteins. Because endotoxin could interfere with use of the subsequent use
255 of purified proteins, we tested the recombinant protein-positive fractions for endotoxin
256 activity and found that endotoxin could not be detected.
257
258 **Immunological activity of the purified rFagag1 and rGFP-Fagag1**
259 To determine if purified rFagag1 and rGFP-Fagag1 have immunological activity,
260 splenocytes from buckwheat crude protein-immunized mice were stimulated with
261 buckwheat crude protein or purified recombinant proteins *in vitro.* We used buckwheat
262 crude protein to determine the optimal amount of allergen (Fig. 6a). Buckwheat crude
263 protein was capable of inducing high levels of expression of IL-13 mRNA in
264 splenocytes at a concentration of >10 μg/mL buckwheat. We next examined the ability
265 of purified rFagag1 and rGFP-Fagag1 to induce IL-13 mRNA expression in splenocytes
266 isolated from buckwheat-immunized mice. In splenocytes stimulated with 10 μg/mL
267 rFagag1 for 72 hr, expression of IL-13 mRNA was significantly higher than what was
268 observed for a negative control (medium) or 10 μg/mL rGFP-Fagag1 (Fig. 6b). The

269 rGFP-Fagag1 treatment induced a small but significant level of up-regulation of IL-13
270 mRNA as compared with the control (Fig. 6b).
271 We next evaluated the ability of rFagag1 to induce the Th2 and Th17 cytokines.
272 Induction of IL-4 and IL-17F facilitates the establishment of Th2 and Th17-based
273 adaptive immune responses. Thus, we examined the ability of rFagag1 to induce IL-4
274 (Fig. 7a) and IL-17F (Fig. 7b) mRNA in splenocytes from buckwheat-immunized mice
275 by real-time qPCR. The cells were cultured as described above with rFagag1 for 72 hr.
276 Our preliminary data indicate that rGFP does not induce IL-4 and IL-17F mRNA
277 expression (data not shown). Expression of IL-4 and IL-17F was elevated in
278 rFagag1-treated splenocytes from buckwheat-immunized mice; however, treatment of
279 splenocytes with buckwheat protein resulted in higher levels of IL-4 and IL-17F than
280 were induced by rFagag1 (Fig. 7)
281
282 **Discussion**
283 Expression and purification of recombinant fusion proteins is a well-accepted
284 approach for production of allergen proteins. Previously, we generated a recombinant
285 NZ9000 strain expressing a GFP fusion with α_{S1} casein, a milk major allergen
286 (Shigemori et al. 2012). In this study, maximal expression of a GFP-Fagag1 fusion in
287 NZ9000-GFP-Fagag1 cells grown at 30°C was achieved with the following culture
288 parameters: OD_{600} of 0.4, induction with 100 ng/mL nisin, and 1-3 hr of incubation.
289 Fluorescence is a convenient way to evaluate protein expression and purification (Niu et
290 al. 2008; Waldo et al. 1999). To take advantage of this, we constructed a GFP fusion
291 expression vector that can be propagated in NZ9000. Based on the results with
292 His-tagged GFP, the vector appears to be generally applicable for the expression of GFP

293 fusion proteins, including GFP-Fagag1. As there was a good correlation between RFU
294 and the amount of rGFP-Fagag1 (data not shown), we were able to optimize expression
295 levels by simply measuring GFP RFU. To test the potential immunological activity of
296 rGFP-Fagag1, we asked if purified rGFP-Fagag1 could induce Th2-mediated allergic
297 responses in splenocytes isolated from buckwheat-immunized mice. Th2 cell-mediated
298 immune responses against "innocuous" antigens play a role in triggering allergic
299 diseases (Romagnani 2004) and are characterized by the prevalent production of IL-4,
300 IL-5, IL-9, and IL-13 (Nguyen and Casale 2011). Here, we showed that splenocytes
301 isolated from buckwheat crude protein-immunized mice and subsequently treated with
302 purified rGFP-Fagag1 for 72 hr did not induce IL-13 mRNA to high levels. Thus,
303 although it induced a modest response, purified rGFP-Fagag1 purified from NZ9000 is
304 unlikely to be useful for studies specifically focused on food-based allergic responses.
305 Our studies using purified rFagag1 show more promise. Treatment with rFagag1
306 up-regulated the expression of IL-4, IL-13 and IL-17F in splenocytes from
307 buckwheat-immunized mice, although not to the same extent that we observed with
308 buckwheat crude protein. The identification of novel helper T (Th) cell subsets, i.e.,
309 IL-17-producing Th cells (Th17 cells) and regulatory T cells (Treg cells), provides new
310 insight into understanding the molecular mechanisms involved in the development of
311 infectious and autoimmune diseases, as well as immune responses, and thus leads to a
312 revision of the classic Th1/Th2 paradigm (Cosmi et al. 2011). Th17 cells may also
313 contribute to the pathogenesis of classically recognized Th2-mediated allergic disorders.
314 IL-17F, which like IL-17 is a member of the IL-17 cytokine family of molecules, binds
315 to IL-17R and has similar pathological activities as compared with IL-17 (Suzuki et al.
316 2007, Vazquez-Tello et al. 2012). IL-17F is derived from a limited set of cell types,

317 including activated $CD4^+$ T cells, basophils, and mast cells. Moreover, IL-17F induces
318 expression of several CXC chemokines and cytokines, including profibrotic cytokines
319 such as IL-6 and TGF-β (Fujie et al. 2012; Takei-Taniguchi et al. 2012; Vazquez-Tello
320 et al. 2012). In a mouse model with increased numbers of neutrophils in the airways,
321 over-expression of IL-17F was shown to have an additive effect on antigen-induced
322 allergic inflammatory responses, such as increased airway hyper-responsiveness, goblet
323 cell hyperplasia, and mucin gene expression, phenotypes that are associated with the
324 pathophysiology of allergic inflammation (Hurst et al.2002; Oda et al. 2005). The
325 symptoms of buckwheat allergies are dependent on how buckwheat is used in a product
326 (i.e. the mode of delivery). Buckwheat products that are ingested induce food
327 allergy-associated symptoms, whereas products such as pillows stuffed with buckwheat
328 chaff can cause nocturnal asthma. The most dangerous allergic reaction to buckwheat is
329 anaphylaxis, which can lead to a fatal shock if left untreated. Importantly, then,
330 understanding the cellular source and targeting cells of the IL-17 cytokine family may
331 provide a basis for elucidating cellular mechanisms that underlie allergic inflammation
332 and improve therapeutic approaches to buckwheat allergies.
333 The immunogenicity of soluble proteins administered orally or intranasally is
334 generally low and can be significantly enhanced either by coupling the protein to a
335 bacterial carrier or by genetic engineering bacteria to produce the target antigen.
336 Food-grade or commensal Gram-positive bacteria constitute an attractive alternative to
337 attenuated pathogenic bacteria for inducing immunity (Wells and Mercenier 2008).
338 More specifically, lactic acid bacteria are an attractive alternative to attenuated
339 pathogens, as they can be good candidates for the development of novel oral vectors for
340 mucosal delivery strategies. Today, sufficient data are available supporting the idea that

lactic acid bacteria, notably lactococci and lactobacilli, are excellent candidates as delivery vectors of therapeutic proteins and thus should be useful in the development of novel preventive and therapeutic strategies. *Lc. lactis* and certain species of lactobacilli possess a number of attractive properties that make them suitable candidates for the development of mucosal vaccines (Mannam et al. 2004).

In conclusion, we have described a convenient method for production of rFagag1 or rGFP-fused Fagag1 with a good protein yield in an advantageous probiotic host. We have demonstrated an assay of immunological activity for rFagag1 in which the recombinant protein shows a high level of allergic activity, including up-regulation of IL-17F expression. rFagag1 purified from NZ9000 might also be useful for significant applications in basic research into food allergic disease. In addition, the strain NZ9000-Fagag1 may be a useful candidate organism for the development of allergic vaccines, particularly oral vaccines. However, the future application of prophylactic and therapeutic strategies based on NZ9000-Fagag1 first requires the completion of a number of studies, including a clear demonstration of efficacy in *in vivo* trials.

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447 **Figure Legends**

448 **Fig. 1.** Schematic representation of constructs used in this study. (a) Map of the pNSH
449 vector, a modified form of pNZ8148. The PnisA promoter and terminator are upstream
450 and downstream of a multiple cloning site (MCS), respectively. The 6x His tag and
451 factor Xa recognition site (FXa) are between the PnisA promoter and the MCS. (b, c, d)
452 Schematic representation of recombinant protein expression vectors. Codon-optimized
453 gene fragments for GFP (714 bp encoding 238 aa) and Fagag1 (1,611 bp encoding 537
454 aa) were excised with restriction enzymes from pCR2.1 and then inserted into the MCS
455 of pNSH. Abbreviations: nisin-inducible promoter, PnisA; ribosome binding site, RBS;
456 Factor Xa recognition site, FXa; multiple cloning site, MCS; terminator, T; replication
457 gene, rep; chloramphenicol acetyltransferase gene, CM.

458 **Fig. 2.** Expression of recombinant proteins in *Lc. lactis* NZ9000. Cell fractions cultured
459 with (+) or without (-) 100 ng/mL nisin for 3 hr were analyzed by SDS-PAGE (a) and
460 western blotting using anti-His-tag Abs (b). Arrows indicate rGFP-Fagag1 (91.7 kDa),
461 rFagag1 (65.4 kDa) and rGFP (30.6 kDa).

462 **Fig. 3.** Confocal laser microscopic analysis of *Lc. lactis* NZ9000 expressing
463 GFP-tagged Fagag1. Nisin-induced NZ9000 cells were analyzed under visible and
464 fluorescent light, and then merged images were generated. *Bar* = 5 μm.

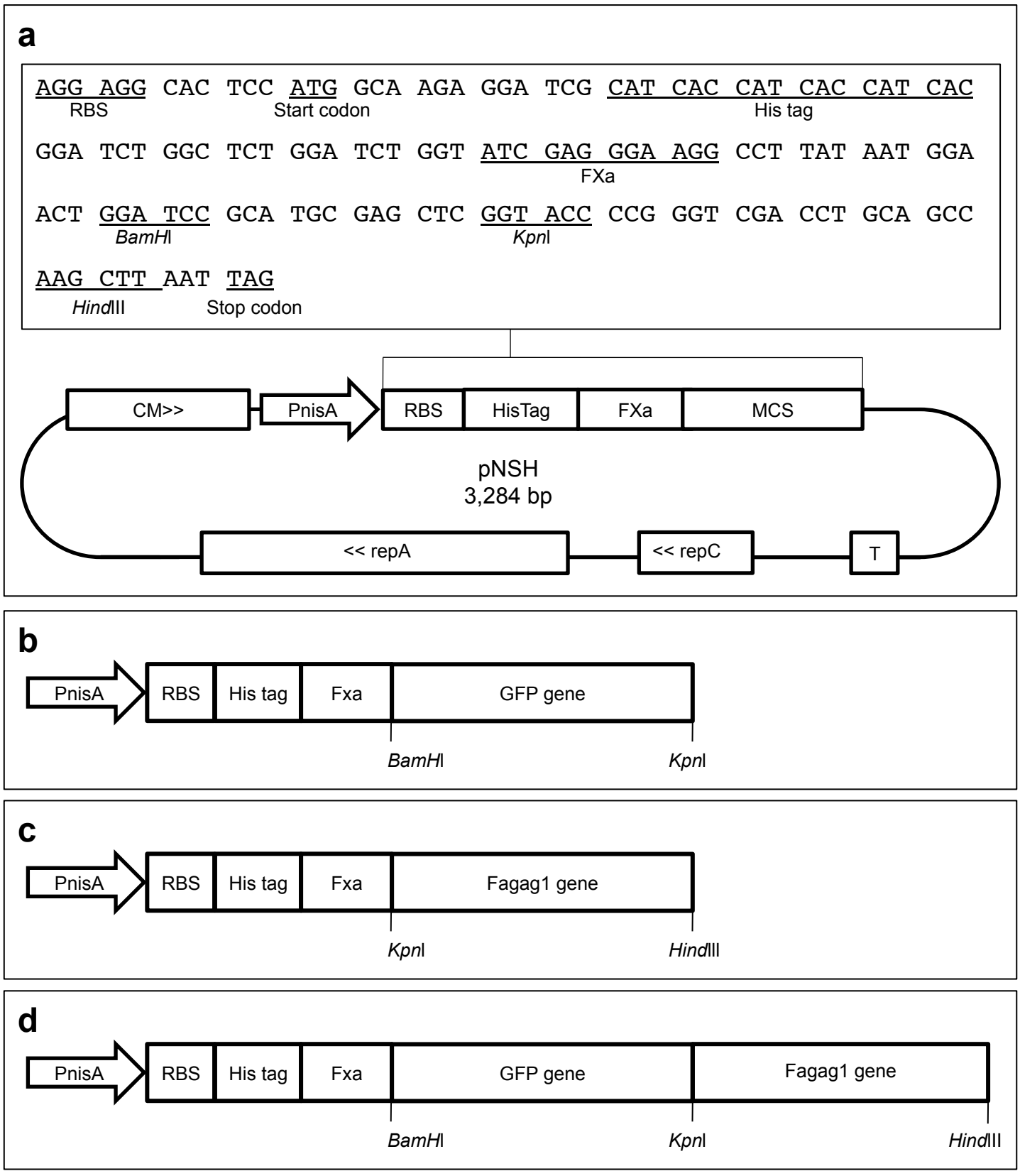
465 **Fig. 4.** Determination of optimal conditions for GFP-Fagag1 expression in *Lc. lactis*.
466 Optimal conditions for GFP-Fagag1 expression in the *Lc. lactis* strain were determined
467 by measuring fluorescence (a, b), growth curves (c) and external pH (d) after induction
468 with nisin at various concentrations and incubation times. Relative fluorescence index:
469 relative fluorescence units (RFU) are displayed as relative values versus blank (water).
470 $*P < 0.05$, $**P < 0.01$ versus vector control (a, b). Data are shown as mean ± SD.

Fig. 5. Purification of recombinant Fagag1 and control proteins from *Lc. lactis* NZ9000. Cell fractions for three recombinant proteins, GFP (a), Fagag1 (b) and GFP-Fagag1 (c) were isolated following induction of expression in the corresponding NZ9000 strains using 100 ng/mL nisin for 3 hr in large scale (3 L), following by protein purification using a His-Trap column. For each strain, the crude lysate before purification, flow-through and eluted fractions were analyzed by SDS-PAGE and western blotting using anti-His-tag Abs. Arrows indicate rGFP (30.6 kDa) (a), rFagag1 (65.4 kDa) (b) and rGFP-Fagag1 (91.7 kDa) (c).

Fig. 6. Immune response of buckwheat crude protein-treated splenocytes to rFagag1 and rGFP-Fagag1. Splenocytes were isolated from buckwheat crude protein-sensitized BALB/c mice and stimulated with or without various concentrations of buckwheat crude protein (a), or 10 μg/mL purified rFagag1 or rGFP-Fagag1 (b). After 72 hr, IL-13 mRNA levels were measured by real-time quantitative PCR analysis. Values represent means and error bars indicate SD of three or four independent experiments. $^{**}P < 0.01$ versus medium control (a). Items indicated with different letters (*i.e.,* a, b, c) were significantly different ($P < 0.05$) (b). Similar results were obtained from at least three different mice.

Fig. 7. Immune response of buckwheat crude protein-treated splenocytes to rFagag1. Splenocytes were isolated from buckwheat crude protein-sensitized BALB/c mice and stimulated with or without 10 μg/mL buckwheat crude protein or purified rFagag1. After 72 hr, IL-4 (a), IL-17F (b) mRNA levels were measured by real-time quantitative PCR analysis. Values represent means and error bars indicate SD of three or four independent experiments. $^{**}P < 0.01$ versus medium control. Similar results were obtained from at least three different mice.

Figure 1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RBS
Start codon
His tag
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FXa
ACT GGA TCC GCA TGC GAG CTC GGT ACC CCG GGT CGA CCT GCA GCC
BamHI
KpnI
AAG CTT AAT TAG
HindIII
Stop codon
CM>>
PnisA
RBS
HisTag
FXa
MCS
pNSH
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<< repA
<< repC
T
b
PnisA
RBS
His tag
Fxa
GFP gene
BamHI
KpnI
c
PnisA
RBS
His tag
Fxa
Fagag1 gene
KpnI
HindIII
d
PnisA
RBS
His tag
Fxa
GFP gene
Fagag1 gene
BamHI
KpnI
HindIII

Figure 2

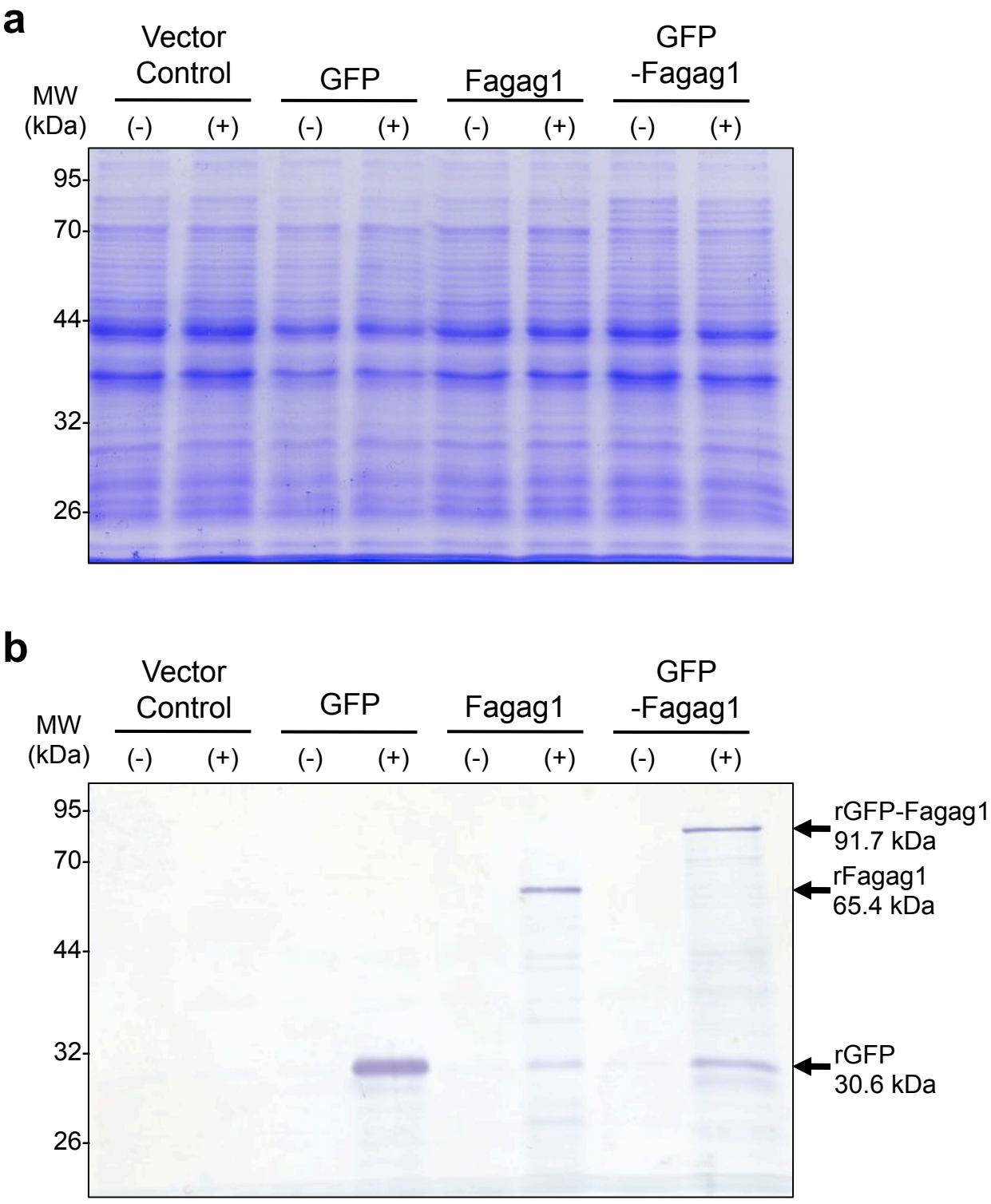
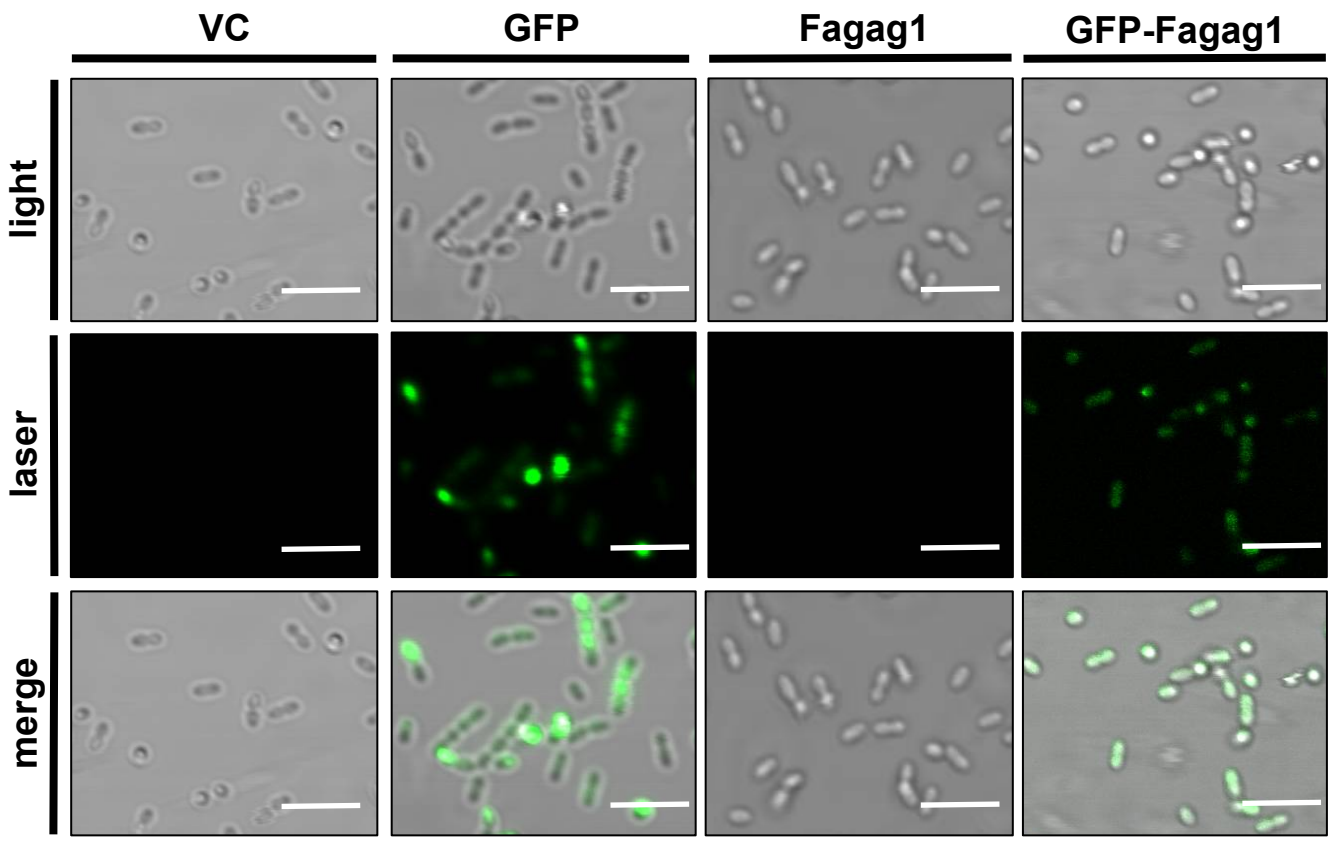

a
Vector Control
GFP
Fagag1
GFP -Fagag1
MW (kDa)
(-) (+) (-) (+) (-) (+) (-) (+)
95
70
44
32
26
b
Vector Control
GFP
Fagag1
GFP -Fagag1
MW (kDa)
(-) (+) (-) (+) (-) (+) (-) (+)
95
70
44
32
26
rGFP-Fagag1
91.7 kDa
rFagag1
65.4 kDa
rGFP
30.6 kDa

Figure 3



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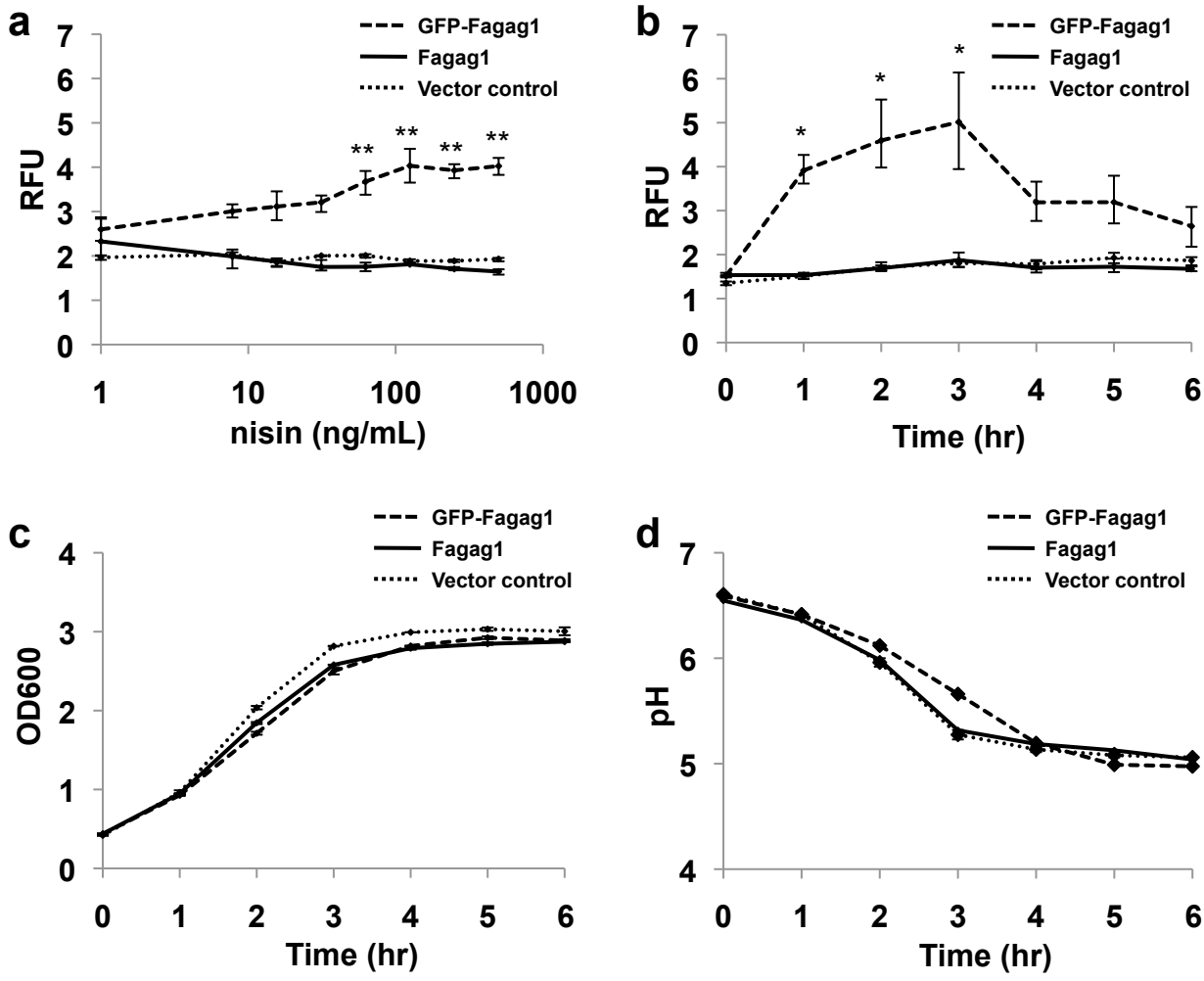

a
GFP-Fagag1
Fagag1
Vector control
RFU
nisin (ng/mL)
b
Time (hr)
c
OD600
d
pH

Figure 5

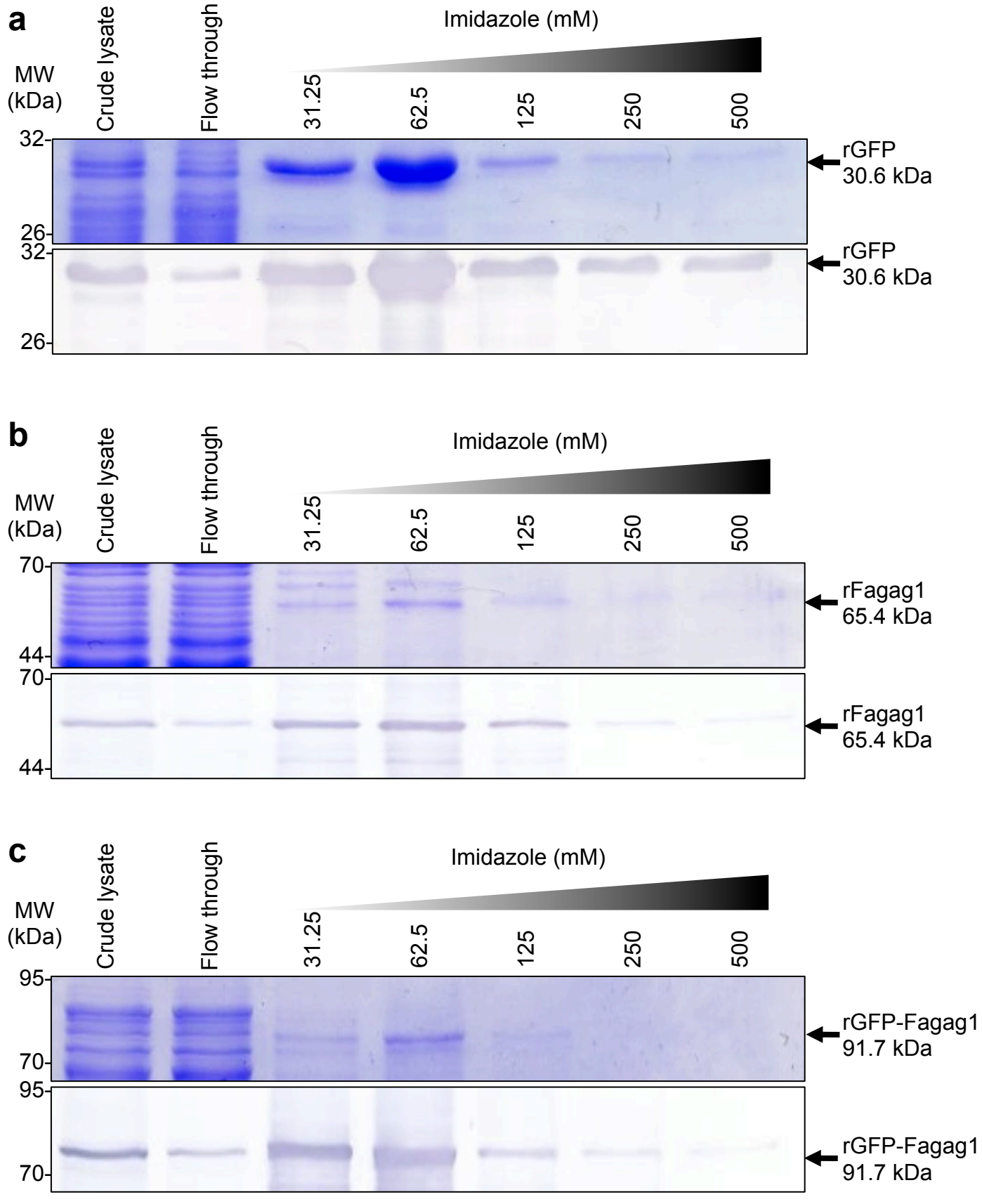

a
MW (kDa)
Crude lysate
Flow through
Imidazole (mM)
31.25
62.5
125
250
500
32
26
32
26
rGFP 30.6 kDa
rGFP 30.6 kDa
b
MW (kDa)
Crude lysate
Flow through
Imidazole (mM)
31.25
62.5
125
250
500
70
44
70
44
rFagag1 65.4 kDa
rFagag1 65.4 kDa
c
MW (kDa)
Crude lysate
Flow through
Imidazole (mM)
31.25
62.5
125
250
500
95
70
95
70
rGFP-Fagag1 91.7 kDa
rGFP-Fagag1 91.7 kDa

Figure 6

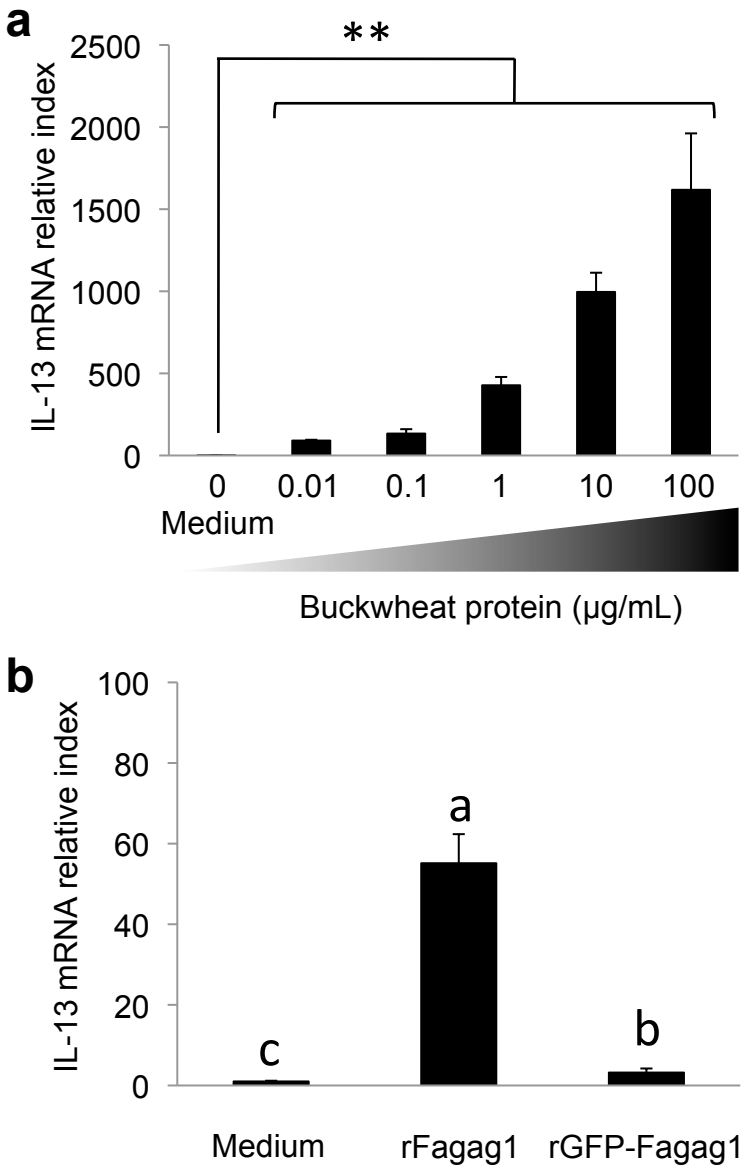
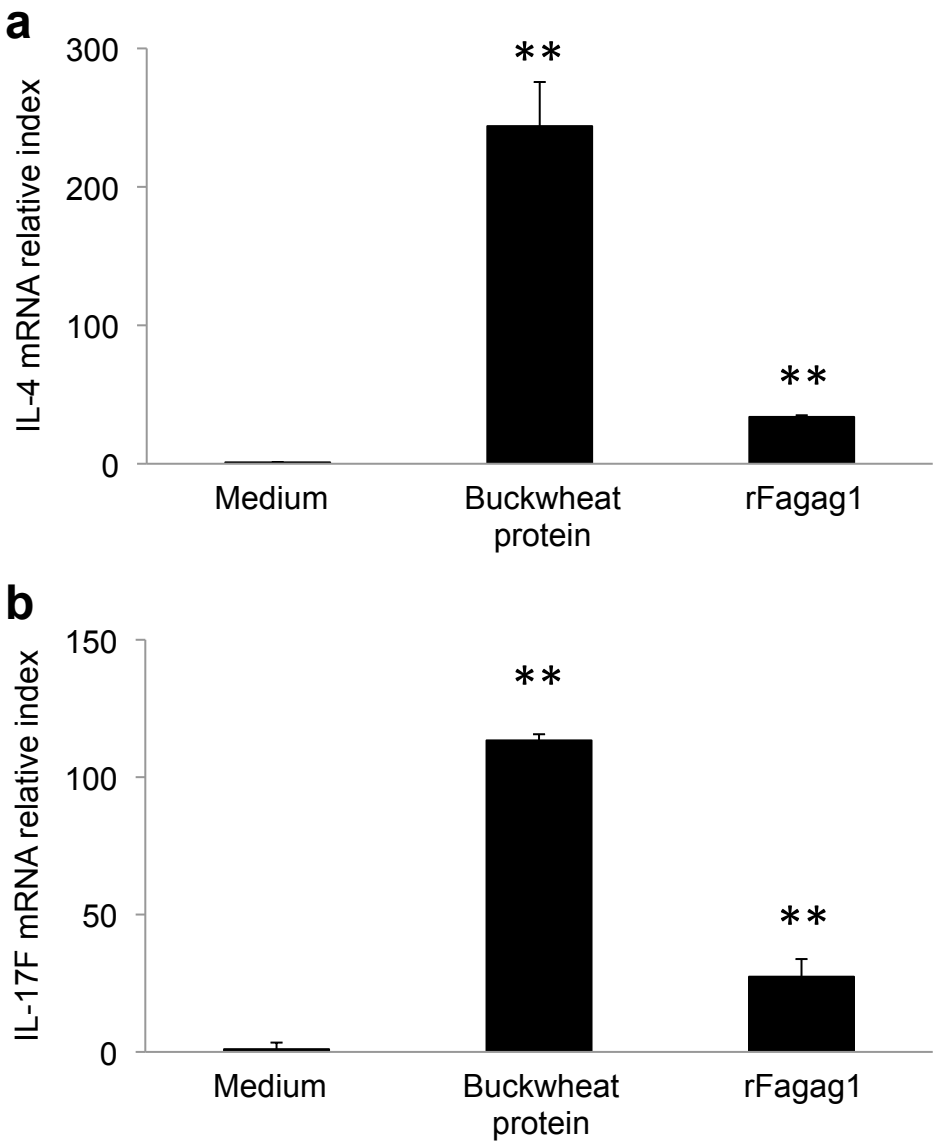

a
**
2500
2000
1500
1000
500
0
IL-13 mRNA relative index
0
0.01
0.1
1
10
100
Medium
Buckwheat protein (μg/mL)
b
100
80
60
40
20
0
IL-13 mRNA relative index
a
b
c
Medium
rFagag1
rGFP-Fagag1

Figure 7


a
IL-4 mRNA relative index
300
200
100
0
**
**
Medium
Buckwheat protein
rFagag1
b
IL-17F mRNA relative index
150
100
50
0
**
**
Medium
Buckwheat protein
rFagag1