

# Activity-based protein profiling of hydrolytic enzymes induced by gibberellic acid in isolated aleurone layers of malting barley

S. Nicolas Daneri-Castro<sup>a</sup>, Balakumaran Chandrasekar<sup>b</sup>, Friederike M. Grosse-Holz<sup>b</sup>, Renier A.L. van der Hoorn<sup>b</sup> and Thomas H. Roberts<sup>a</sup>

<sup>a</sup>Plant Breeding Institute, Faculty of Agriculture and Environment, University of Sydney, 1 Central Ave, Eveleigh NSW 2015, Australia.

<sup>b</sup>The Plant Chemetics Laboratory, Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, U.K.

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## Abstract

During barley germination, the aleurone layer secretes most of the enzymes required to degrade the endosperm, many of which are yet to be characterized. We used activity-based protein profiling (ABPP) to detect a range of active enzymes extracted from aleurone layers isolated from grains of a commercial malting barley variety incubated with or without gibberellic acid (GA). Enzymes found to be induced by GA were putative aleurains, cathepsin-B-like proteases and serine hydrolases. By using an inhibitory sugar panel, a specific active retaining  $\beta$ -glycosidase in the barley aleurone was identified as a xylanase. Our results show that ABPP can be used rapidly to identify a variety of active enzyme isoforms in cereal aleurone without the need for enzyme purification.

**Abbreviations:** ABA, abscisic acid; ABPP, activity-based protein profiling; GA, gibberellic acid; PLCP, papain-like cysteine protease; VPE, vacuolar processing enzyme.

## Introduction

Malting is the controlled germination of the mature cereal grain (most commonly barley) to partially degrade the products stored in the endosperm, thereby releasing compounds that will be used in the brewing process or in food manufacturing [1]. Most of the degrading enzymes required for germination are produced and secreted by the aleurone layer, which surrounds the starchy endosperm. The production and secretion of these enzymes are

triggered by gibberellic acid (GA) secreted by the embryo during germination [2]. The activity of the aleurone layer cells is tightly regulated by the concentration ratio between GA and abscisic acid (ABA), with ABA counteracting the effects of GA [3]. A major goal of barley maltsters is to predict the behaviour of the grain during germination in order to optimize the whole malting process—but this remains a key challenge [4]. Considering the importance of the aleurone layer during germination, new techniques to advance the study of this tissue will provide valuable information to understand the behaviour of the whole barley grain during germination.

Most studies of isolated aleurone layers exposed to ABA and GA have focused on changes in gene expression, protein presence/absence and the abundance of certain enzymes [5, 6]. Insights have been gained into the key metabolic processes taking place in the aleurone layer during germination, but relatively little is known about the presence or abundance of enzyme isoforms in their active form. This knowledge is crucial, as the presence of an enzyme often does not relate to its activity [7]. By the use of active site-directed chemical probes that covalently bind their targets, the functional state of enzymes can be evaluated directly in a protein sample [8]. This technique is known as activity-based protein profiling (ABPP), which was originally developed for applications in medical biochemistry [9], but has been successfully translated into the study of enzyme activities in plants [10]. One of the major advantages of ABPP is that, by using distinct probes, specific subsets (families) of enzymes can be evaluated in the same sample [11].

We employed ABPP to study enzyme activities in isolated aleurone layers of barley incubated with and without GA. We used the commercial malting variety Gairdner to optimise the relevance of our results to industry.

## Materials and Methods

### Aleurone layer extraction and hormone incubation

A total of 120 quarter grains (cv. Gairdner) were prepared for each aleurone layer extraction. From dry whole grains, the embryo-containing half followed by the quarter containing the ventral furrow were removed and discarded. Under sterile conditions, the remaining quarter grains were surface-sterilized with 2% (w/v) NaOCl for 30 min, followed

by four washes with sterile Milli-Q water. The quarter grains were then imbibed in Milli-Q water containing cefatoxime (150 µg/ml) and nystatin (50 µg/ml) for 72 h at room temperature, while covered in aluminium foil.

After imbibition, aleurone layers were isolated from the quarter grains. The endosperm was removed by scraping with a scalpel (N°3) and fine-pointed forceps. A small volume of Milli-Q water was then added with the forceps to the quarter grain and any remains of endosperm gently scraped away. The aleurone layers were carefully peeled away from the husk and the testa with two Swiss-style forceps (N° 4 and 5). The isolated layers were briefly washed by submerging them in Milli-Q water and then transferred to a small petri dish (60 mm dia.) with 3 ml of Milli-Q water.

The isolated aleurone layers were distributed equally into three petri dishes, each used for a different incubation condition: aleurone layers shortly after being extracted (Initial); aleurone layers incubated in 2 ml of incubation media (10 mM CaCl<sub>2</sub>, 5 µM ABA) for 48 h (ABA-GA); and aleurone layers incubated in 2 ml of incubation media with 13 µM GA for 48 h (ABA+GA). For sample preparation, the layers were dried on a paper towel to remove any excess of incubation media. Using fine-point forceps the layers were then transferred to a microcentrifuge tube with a secured screw cap and were snap frozen with liquid nitrogen. The samples were stored at -80°C for subsequent experiments.

#### Protein extraction and determination of protein concentration

The aleurone layers were homogenized using a small ceramic mortar and pestle by adding a small volume of liquid nitrogen and carefully grinding until a fine powder was obtained. The powder was resuspended and homogenized in the mortar with 1.2 ml of ice-cold extraction buffer (50 mM sodium acetate, pH 5.5, 5 mM DTT) and transferred to a 1.5-ml microcentrifuge tube. The protein extraction was performed for 45 min with soft agitation on an orbital rocker in a cold room (4°C). The microcentrifuge tube was then centrifuged at 13,000 rpm at 4°C for 10 min. The supernatant containing the soluble proteins of the aleurone layers was carefully transferred to a new microcentrifuge tube and stored on ice.

Protein concentration was determined with the DC Protein Assay (BioRad) following the manufacturer's instructions except that all volumes recommended in the protocol were reduced by a factor of 2.5 to reduce the volume of sample required.

#### Probe labeling of extracted proteins

Labeling was performed in a 50- $\mu$ l total volume. A total of 49  $\mu$ l of a suspension of aleurone layer proteins (~30  $\mu$ g of soluble protein) was incubated with 1  $\mu$ l ABPP probe dissolved in DMSO (2  $\mu$ M FY01, JOGDA1, JOPD1, JJB70, MV201, MV201, or 0.5  $\mu$ M FP-Rh) for various times (Table 1) in the dark. For the inhibitors, a total of 45  $\mu$ l of aleurone layer proteins (~29  $\mu$ g of soluble protein) was preincubated with 100  $\mu$ M E-64 or Ac-YVAD-cmk or DCI (Table 1) for 30 min at room temperature. The samples were then labeled with the corresponding probes as previously described. For the no-probe controls, 1  $\mu$ l of DMSO was added instead of the probe. For the monosaccharides panel, 39  $\mu$ l of aleurone layer proteins was preincubated with 10  $\mu$ l of the various monosaccharides (1.0 M) for 30 min at room temperature followed by probe labeling as previously described.

#### Analysis of labeled proteins

The labeling reactions (50  $\mu$ l) were stopped by adding 15  $\mu$ l of 4X gel-loading buffer (200 mM Tris-HCl (pH 6.8), 400 mM DTT, 8% SDS, 0.04% bromophenol blue, 50% glycerol) and heating at 95°C for 5 min. The labeled proteins (13  $\mu$ l, ~6  $\mu$ g) were separated on 12% (v/v) midi protein gels (8 x 13 cm, 26 wells) at 200 V for 1 h in the dark. Labeled proteins were detected on the protein gels with a Typhoon FLA 9400 (Amersham Biosciences/ GE Healthcare) flatbed scanner using an excitation wavelength of 532 nm and a 580-nm band-pass filter (580BP30) for all the probes except JJB70, for which an excitation wavelength of 488 nm and a 520-nm band-pass filter (520BP40) were used. After the fluorescence signals were obtained, the proteins were stained with Coomassie Brilliant Blue (CBB) solution (5% aluminium sulfate hydrate, 10% ethanol, 0.02% Coomassie G-250, 8% phosphoric acid) to display the abundant proteins present.

## Results and Discussion

### Initial assessment of labeling by various probes of aleurone layer proteins

Labeling by the different probes targeting various enzyme groups (Table 1) was evaluated on a single sample of aleurone layer proteins. Aleurone layers treated with ABA and GA for 48 h were chosen since they were expected to have the highest content of active enzymes induced by GA. The influence of the pH of the protein extraction buffer was also evaluated by testing an acidic buffer (pH 5.5) and a neutral buffer (pH 7.5). The acidic extraction buffer resulted in a larger number of signals than the neutral buffer, especially with probes against proteases (data not shown). Therefore, the acidic extraction buffer was used to extract proteins from all the aleurone layer samples used in subsequent experiments.

The signals obtained with FY01 (Fig. 1), which targets mostly aleurain-like proteases, were similar to the signals obtained with MV202 and MV201, which target all papain-like cysteine proteases (PLCPs) [12]. Thus FY01 was chosen for subsequent experiments. Signals were also detected for JOGDA1, which targets cathepsin-B-like proteases, and JOPD1, which targets vacuolar processing enzymes [12]. Multiple strong signals were displayed upon labeling with JJB70, which targets retaining  $\beta$ -glycosidases [13] and FP-Rh, which targets serine hydrolases [14]. For MVB072, which targets the catalytic beta subunit of the proteasome, clear signals were observed (Fig. 1) with a mobility corresponding to the expected molecular weight (~25 kDa) [15].

### Characterization of protease activity in aleurone layers incubated with ABA, with or without GA

The recent availability of probes able to target specific groups of proteases represents an advance in the study of the hydrolytic enzymes triggered by GA. The samples used for labeling were: aleurone layers without incubation (Initial); aleurone layers incubated with ABA without GA for 48 h (ABA-GA); aleurone layers incubated with ABA and GA for 48 h (ABA+GA). Controls used were a mixture of all three samples labeled with and without probe or preincubated with inhibitor before incubation with the probe. The labeled proteins were separated on reducing SDS-PAGE and analysed by fluorescence scanning and CBB staining.

149

150 We detected a strongly increased FY01 labeling upon GA treatment (Fig. 2A) consistent with  
151 the production of active aleurain-like proteases (~32 kDa) induced by GA, as previously  
152 observed with aleurone layers derived from the hull-less barley variety, Himalaya [16]. We  
153 believe this is the first time the induction of active aleurain-like proteases has been  
154 observed in aleurone layers from a commercial malting variety.

155

156 An induction of the activity of cathepsin-B-like proteases was observed with the JOGDA1  
157 probe upon incubation of aleurone layers with GA for 48 h (Fig. 2B). This concurs with  
158 previous evidence of the induced expression of the *CatB* gene in isolated aleurone layers  
159 incubated with GA [17]. The observed signal band corresponds to the molecular weight of  
160 the mature form of this cysteine protease (~34 kDa). These results show the potential and  
161 versatility of the use of ABPP to characterize specific groups of proteases in barley grain  
162 under different conditions.

163

164 Signals with JOPD1, which targets vacuolar processing enzymes (VPEs, ~40-43 kDa) [18],  
165 provided evidence for an induction of activity upon incubation of aleurone layers without  
166 GA (Fig. 3A). This induction was greater and with a different profile than that obtained upon  
167 incubation with GA. The role of VPEs in the induction of programmed cell death in barley  
168 has been previously described for the maturing grain [19]. Therefore, our findings contradict  
169 the expected effect of ABA, which delays the programmed cell death response of the  
170 aleurone layer cells [20]. The apparent suppression of VPE activity due to GA treatment and  
171 the lower molecular weight of the induced bands might imply that there is proteolytic  
172 degradation in the GA-treated sample, due to induction of proteases and cell death. Our  
173 observations show the potential of this technique to complement studies of programmed  
174 cell death (PCD) in aleurone layer cells.

175

176 Analysis of the signals obtained with FP-Rh (Fig. 3B) is challenging since this probe targets  
177 many serine hydrolases [14], a large class of enzymes including proteases, esterases, lipases  
178 and acyltransferases [21]. For the discrimination of specific enzymes and to provide  
179 evidence for the specificity of the labeling, the experiment was complemented with the use

of the serine hydrolase inhibitor 3,4-dichloroisocoumarin (DCI). A GA-induced band at ~30 kDa (Fig. 3B, filled arrowhead), was considerably decreased by preincubation with DCI, indicating that this enzyme can be inhibited by DCI. Therefore, we infer that this signal corresponds to a serine hydrolase induced in the aleurone layers by GA. Many additional signals did not respond to GA treatment and one signal band was suppressed by GA treatment (Fig. 3B, empty arrowhead).

#### Identification of glycosidase activity in aleurone layers using ABPP

The probe JJB70 was used to detect the activity of the retaining  $\beta$ -glycosidases present among the proteins obtained from the aleurone layer samples incubated with or without GA (Fig. 4A). Many signals were observed, indicating the presence of various active retaining  $\beta$ -glycosidases among the proteins of the aleurone layers under the different treatments. Unexpectedly, striking differences in their expression upon incubation with GA were not observed.

A simple way to annotate glycosidase signals is to perform labeling in the presence of monosaccharides (Chandrasekar B., unpublished). We used three monosaccharides to evaluate the glycosidases present in the control aleurone layers (not incubated with hormones). We chose this sample as it showed a different band profile compared to the aleurone layers incubated with or without GA.

A single ABPP band corresponding to a protein of ~65 kDa had greatly reduced intensity following pre-incubation with xylose (Fig. 4B). This suggests that this glycosidase can be inhibited by xylose. A likely candidate is a xylanase, which is required for the degradation of the 1,4-xylan backbone of arabinoxylans, the major cell wall constituent of the aleurone layer cells [22]. Studies on xylanase expression in aleurone layers of germinating barley grain suggested that the mature and active form (~34 kDa) was derived after successive post-translational modifications of a precursor polypeptide (~61.5 kDa) [23]. However, it was later found that the xylanase precursor polypeptide was an active enzyme, which could degrade xylan polymers *in vitro* [24]. Our observations would, therefore, confirm the latter, suggesting that the putative xylanase would correspond to an active form of the precursor

polypeptide. These results provide evidence that the use of this probe could advance further investigations into cell wall-degrading enzymes produced by aleurone layers.

## Conclusion

Our results demonstrate the potential of ABPP to characterize and profile enzyme activities induced (and suppressed) by GA in barley aleurone layers. The induction of activity of aleurain-like proteases, cathepsin-B-like proteases and serine hydrolases was observed by the incubation of aleurone layers with ABA and GA for 48 h. Induction of the activity of vacuolar processing enzymes was also observed by the incubation with ABA and GA for 48 h, but the induction was stronger in the absence of GA. No considerable induction of the activity of retaining  $\beta$ -glycosidases was observed by the incubation with ABA, with or without GA. A decrease in the activity of a specific  $\beta$ -glycosidase was observed by pre-incubation with xylose before the labeling reaction, suggesting that the product of this enzyme is xylose.

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## Author contributions

RALH and THR conceived and designed the experiments. SND-C and BC performed the experiments. SND-C and FMGH analyzed the data and wrote the manuscript with assistance from RALH and THR.

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## Tables

**Table 1. Activity-based fluorescent probes, inhibitors and incubation times used to characterize barley aleurone enzyme activities in this study.**

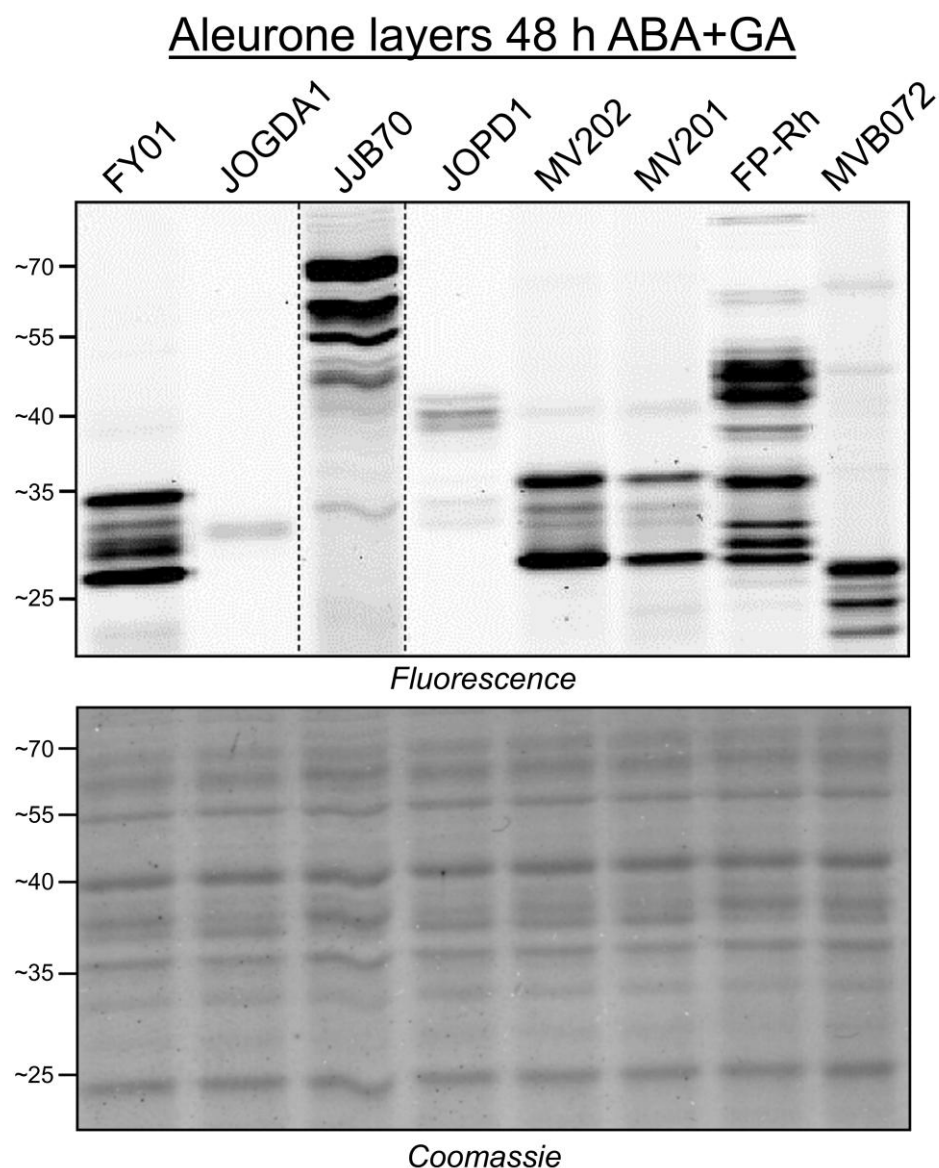
Fluorescent probe	Target enzymes	Enzyme inhibitor	Incubation time (h)	Reference
FY01	Aleurains <sup>a</sup>	E-64 <sup>b</sup>	3	[12]
JOGDA1	Cathepsin-B <sup>a</sup>	E-64 <sup>b</sup>	3	[12]
JOPD1	VPEs <sup>c</sup>	Ac-YVAD-cmk	3	[12]
JJB70	$\beta$ -Glycosidases	N/A	1	[13]
MV202	All PLCPs	N/A	3	[25]
MV201	All PLCPs	N/A	3	[25]
FP-Rh	Serine hydrolases	DCI	1	[14]
MVB072	Proteasome	N/A	2	[26]

<sup>a</sup> subset of enzymes from the papain-like cysteine proteases (PLCPs).

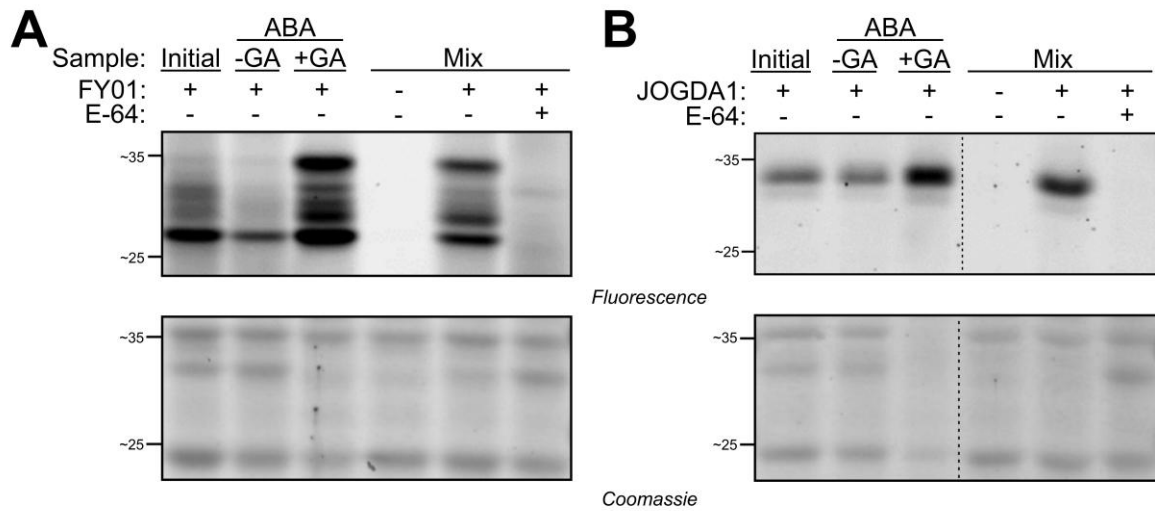
<sup>b</sup> trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane.

<sup>c</sup> vacuolar processing enzymes.

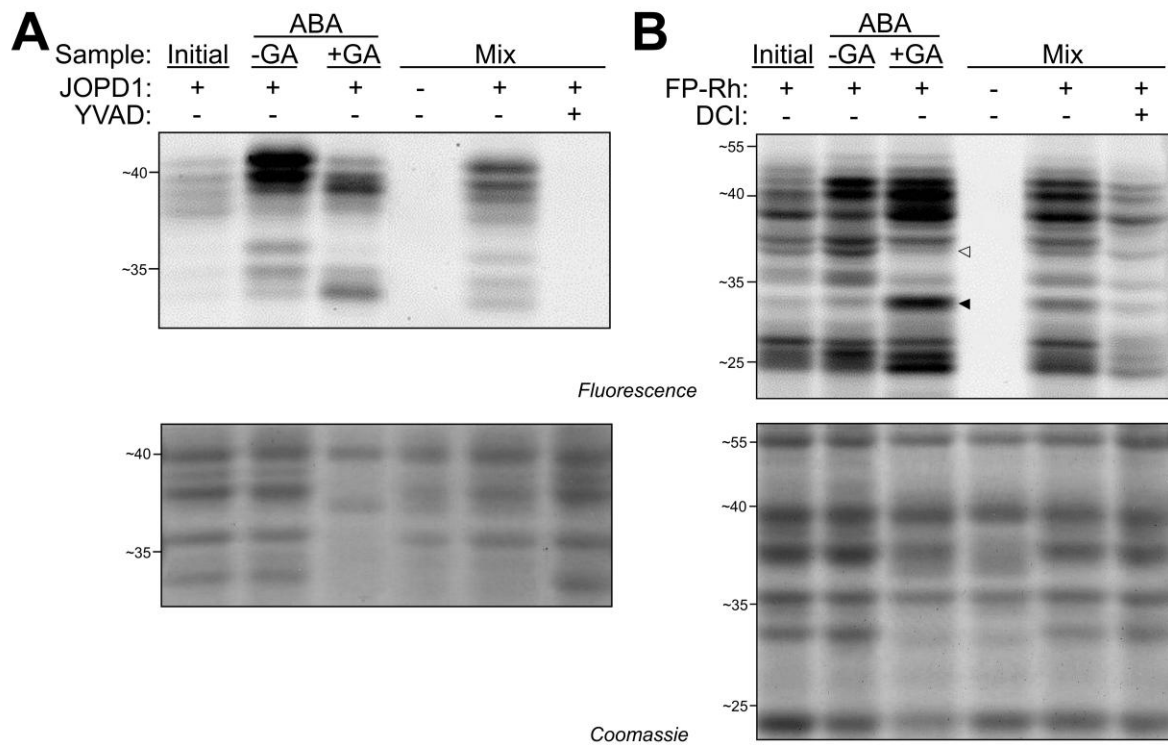
N/A, not applicable



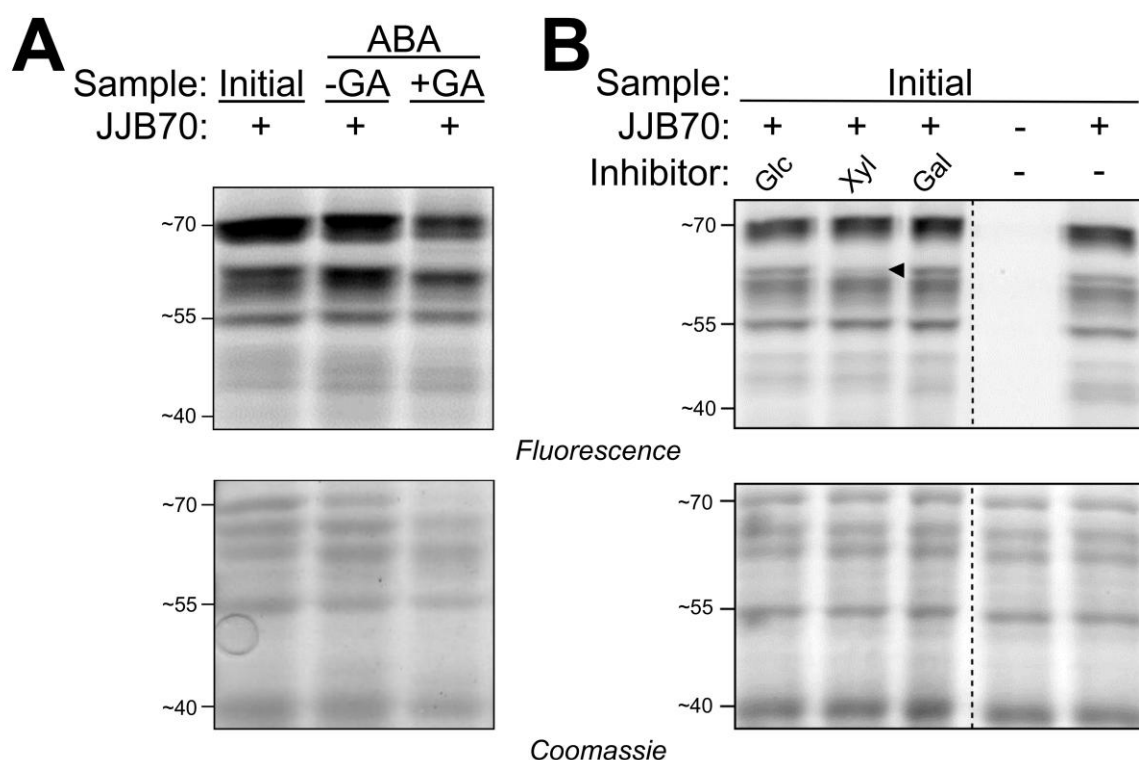
**Figure 1. Initial assessment of various ABPP probes against proteins of aleurone layers incubated with ABA and GA for 48 h.** Proteins were extracted with extraction buffer (50 mM NaAc pH 5.5, 5 mM DTT) and labeled with a range of probes with various incubation times (Table 1). The signals were obtained with an emission wavelength of 580 nm (Cy3) except for JJB70, which was obtained with an emission wavelength of 520 nm (Cy2).



**Figure 2. Cysteine protease activities of barley aleurone layers evaluated with PLCP family-specific ABPP probes.** Proteins from aleurone layers were labeled with probes that target specific families of PLCPs. Enzyme specificities of the probes: A, 2  $\mu$ M FY01, aleurain-like proteases. B, 2  $\mu$ M JOGDA1, cathepsin-B-like proteases. 100  $\mu$ M E-64 was used as the inhibitor for both probes. Mix, mixture of the Initial, ABA-GA and ABA+GA samples. The results shown are representative of three biological replicates.



**Figure 3. Activities of vacuolar proteases and serine hydrolases in barley aleurone layers evaluated with family-specific ABPP probes.** Proteins from aleurone layer samples were labeled with two probes with different enzyme specificities. A, 2  $\mu$ M JOPD1, which targets vacuolar processing enzymes, using the inhibitor 100  $\mu$ M Ac-YVAD-cmk. B, 0.5  $\mu$ M FP-Rh, which targets serine hydrolases, using the inhibitor 100  $\mu$ M DCI (3,4-dichloroisocoumarin). Mix, mixture of the Initial, ABA-GA and ABA+GA samples. Filled arrowhead, GA-induced signal. Empty arrowhead, GA-suppressed signal. The results shown are representative of three biological replicates.



**Figure 4. Evaluation of the activities of glycosidases in barley aleurone layers and of glycosidase inhibition with a monosaccharide panel.** A, The proteins from the aleurone layer samples were labeled with 2  $\mu$ M JJB70, which targets active retaining  $\beta$ -glycosidases. The results shown are representative of three biological replicates. B, Various monosaccharides solutions (200  $\mu$ M) were tested for their ability to inhibit ABPP labeling with the probe JJB70: Glc: glucose; Xyl: xylose; Gal: galactose.