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Diversity and Relative Levels of Actinidin, Kiwellin, and Thaumatin-Like Allergens in 15 Varieties of Kiwifruit (*Actinidia*)

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Supporting Information

ABSTRACT: In the last 30 years the incidence of kiwifruit allergy has increased with the three major allergenic proteins being identified as actinidin, kiwellin, and thaumatin-like protein (TLP). We report wide variation in the levels of actinidin and TLP in 15 kiwifruit varieties from the four most widely cultivated *Actinidia* species. Acidic and basic isoforms of actinidin were identified in *Actinidia deliciosa* 'Hayward' and *Actinidia arguta* 'Hortgem Tahi', while only a basic isoform of actinidin was identified in *Actinidia chinensis* 'Hort16A'. One isoform each of kiwellin and TLP were identified in ripe fruit. The cysteine protease activity of actinidin correlated with protein levels in all species except *A. arguta*. Protein modeling suggested that modifications to the S2 binding pocket influenced substrate specificity of the *A. arguta* enzyme. Our results indicate that care is necessary when extrapolating allergenicity results from single varieties to others within the same and between different *Actinidia* species.

KEYWORDS: *Actinidia, actinidin, allergen, kiwellin, kiwifruit, thaumatin*

INTRODUCTION

Food allergies affect as many as 8% of young children and 2% of adults in westernized countries, with allergy to any specific fruit generally being found to affect <1% of adults (reviewed in Zuidmeer et al.[1]). Allergic reactions to kiwifruit (*Actinidia* spp. Lindl.) have been reported for over 30 years.[2] Clinical symptoms are typically localized to the oral mucosa; however, severe anaphylactic reactions are also observed (reviewed in Lucas et al.[3]). As kiwifruit's availability and consumption has increased since the 1970s, an increase in the incidence of kiwifruit allergy has been noted, presumably due to increased contact.[4] This increase is of clinical concern, as kiwifruit allergy has frequently been associated with allergy to other fruit and foods,[5,6] pollen,[7,8] and in latex-fruit syndrome.[9,10]

A large number of studies have been conducted on protein extracts from *Actinidia deliciosa* fruit to identify the IgE-binding components.[7,11−19] A range of IgE-binding proteins have been identified and named by the Allergen Nomenclature Subcommittee of the International Union of Immunological Societies (http://www.allergen.org) including actinidin (Act d 1), a thaumatin-like protein (TLP, Act d 2), a 40 kDa protein (Act d 3), cystatin (Act d 4), kiwellin (Act d 5), pectin methylesterase inhibitor (Act d 6), pectin methylesterase (Act d 7), Bet v1-related proteins (Act d 8), profilin (Act d 9), lipid transfer protein (Act d 10), major latex protein (Act d 11), and chitinase (Act d chitinase). Of these proteins, actinidin, kiwellin, and TLP have received the most scientific attention. The importance of each allergen to allergic patients can vary significantly, for example, no specific IgE recognition was found for Act d 6 when testing 237 patients' sera.[20] Act d 8 together with Act d 9 are markers for pollen-associated kiwifruit allergy, while Act d 1 is usually linked with kiwifruit monosensitization.[20]

Actinidin is reported to be the major allergen in *A. deliciosa* fruit extracts in some[13,15,18,21] but not all human populations.[5,12,17] It is recorded as an allergen in other *Actinidia* species, e.g., *Actinidia chinensis* (Act c 1), *Actinidia arguta* (Act a 1), and *A. eriantha* (Act e 1) in some databases (e.g., www.allergome.org). Actinidin is a member of the papain subfamily of cysteine proteases that includes papain and chymopapain from papaya (*Carica papaya*), ananain and bromelain from pineapple (*Ananas comosus*), and aleurain from barley (*Hordeum vulgare*). Recently, the enzyme's cysteine protease (CP) activity has been reported to have a beneficial effect on human health by enhancing the gastric digestion of food proteins.[22−24] Actinidin is the most abundant protein in *A. deliciosa* 'Hayward' fruit, but it is not found in the fruit of some *A. chinensis* cultivars, e.g., gold-fleshed 'Hort16A'.[25−27] The protein is synthesized as a zymogen and then processed to an active enzyme by removal an endoplasmic reticulum (ER) targeting signal peptide and N- and C-propeptides. The calculated molecular weight is ∼24 kDa; however, the protein has been reported to run on 1-D SDS PAGE from 24 to 30 kDa depending on conditions. *In planta*, the physiological function of the enzyme is largely unknown, although it has been implicated in pathogen defense[28] and processing of specific proteins, e.g., kiwellin[26] and a class IV chitinase.[29]

Kiwellin is specifically recognized by IgE of some patients allergic to kiwifruit.[16,30−32] It is a cysteine-rich protein that has been shown to be a major protein component of the fruit of both *A. deliciosa* and *A. chinensis*. It has a calculated molecular

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weight of ~20 kDa, although in 1-D SDS PAGE it can run as a band at 28 kDa.[31] Studies by Tuppo et al.[26] have shown that kiwellin is a modular protein with two domains—a 4 kDa N-terminal domain called kissper and a larger C-terminal domain called KiTH. Cleavage of kiwellin to kissper and KiTH can be mediated by actinidin in vitro.[26] No biological function has been ascribed to kiwellin; however, the kissper domain shows pH-dependent and voltage-gated pore-forming activity in lipid membranes.[33]

TLP was identified as a kiwifruit allergen in *A. deliciosa* by Gavrovic-Jankulovic et al.[21] and verified in a number of later studies.[14−16,18,30,34] The protein, with a molecular mass of 21−24 kDa, was subsequently identified as an allergen in *A. chinensis*.[14] TLPs are pathogenesis-related (PR-5) proteins that contain 16 highly conserved cysteine residues. Kiwifruit TLP has been shown to have antifungal activity in vitro.[21]

In the past 10 years the number of kiwifruit cultivars commercially released has significantly increased. Essentially, all these new cultivars are derived from just 4 of the >60 species present within the *Actinidia* genus. *A. deliciosa* fruit (including the predominant 'Hayward' cultivar) are typically large (~100 g) and green-fleshed; *A. chinensis* fruit are also large and can be green-, yellow- ('Hort16A'), or red-fleshed ('Hongyang'); *A. arguta* fruit are small (<25 g), hairless, and sweet,[35] while *A. eriantha* fruit are small, hairy, high in vitamin C, and potentially easy to peel.[36] In this paper we characterize the diversity and relative levels of actinidin, kiwellin, and TLP proteins in 15 different varieties of kiwifruit selected from these four commonly cultivated kiwifruit species.

MATERIALS AND METHODS

Plant Material and Tissue Collection. All kiwifruit plants were grown on the PFR orchard at Te Puke, New Zealand. The individual plant from which fruit was harvested is indicated in brackets for each of the 15 kiwifruit varieties tested: *A. deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson 'Hayward' (43-02-02c), 'Bruno' (14e-10-15b), and 'Qinmei' (21-19-04b); *A. eriantha* Benth. GPA (11-04-18a), GPB (11-03-07a), PPA (11-05-04a), and PPB (11-05-04j); *A. chinensis* (Planch.) 'Hort16A' (43-05-03a), 'Lushan-xiang' (35-03-02a), Jin Feng (43-05-05b), and 'Hongyang' (39-08-09b); and *A. arguta* Sieb. et Zucc. Planch. ex Miq 'Hortgem Wha' (21-06-7a), 'Hortgem Tahi' (21-06-04c), 'Hortgem Toru' (21-06-02a), and 'Hortgem Rua' (12w-01-02c). Detailed fruit descriptions for each variety can be found for 'Hayward', 'Bruno', 'Hort16A', and 'Jin Feng' in Ferguson and Huang,[37] for 'Qinmei', 'Lushan-xiang' and 'Hongyang' in Xiao,[38] and for *A. arguta* 'Hortgem Tahi', 'Hortgem Rua', 'Hortgem Toru', and 'Hortgem Wha' in Williams et al.[35] The *A. eriantha* varieties GPA, GPB, PPA, and PPB are breeding selections that have been used in development of peelable kiwifruit varieties.[36] PPA and PPB are full siblings; 'HortGem Tahi' and 'HortGem Wha' are half siblings; otherwise, the varieties are not closely related genetically.

After harvest, all kiwifruit were held at room temperature until they reached eating ripeness (firmness less than 10 N) as measured by a penetrometer with a 7.5 mm head (Effegi, Alfonsine, Italy). Samples of whole fruit were obtained by taking equatorial slices from at least 10 representative fruit. Core, inner pericarp, outer pericarp, and peel samples were taken from six representative fruit. Ripe fruit of pear (*Pyrus communis*), table grape (*Vitis vinifera*), orange (*Citrus sinensis*), banana (*Musa acuminata*), tomato (*Lycopersicon esculentum*), plum (*Prunus domestica*), avocado (*Persea americana*) 'Hass', and apple (*Malus domestica*) 'Royal Gala' were purchased from a local supermarket and sampled when eating ripe. All tissues were snap frozen in liquid nitrogen and frozen at −80 °C until required.

Antibody Production. The mature open reading frame of kiwellin (*Kwl1-Ae2*, without the ER targeting signal, see Table S1, Supporting Information) was amplified by PCR using primers RA104 5′-ACGGGATCCG CGCCTTCGGG GGCCTCCATC-3′ and RA123 5′-TCCAAGCTTT TACGCCATGG ACCAAGTAA-3′. The insert was excised with *Bam*HI and *Hin*dIII (underlined in the primers) and ligated into corresponding sites of the pET-30a(+) vector (Novagen, Madison, WI). pET-Kwl1 was transformed into *E. coli* BL21 (DE3) Codon Plus RIL cells and recombinant His-tagged protein purified by Ni-affinity chromatography and gel filtration chromatography.[39] Purified soluble recombinant KWL1 protein was used to raise polyclonal antibodies in rabbits (AgResearch, Ruakura, NZ).

Purified native TLP1 protein from *A. deliciosa* fruit extracts was obtained as a gift from Merima Bublin, Medical University of Vienna, Austria. The protein had been purified as described in Bublin et al.[16] and was used to raise a polyclonal antibody in rabbits (AgResearch). The actinidin polyclonal antibody was raised to purified recombinant ACT1a protein as described in Nieuwenhuizen et al.[27] The actinidin antibody has previously been reported to cross-react with equal efficiency to purified recombinant ACT1a, 2a, 3a, and 4a proteins.[27]

Protein Extraction, 1-D SDS PAGE, and Western Blotting. Frozen fruit tissues (700 mg) were ground to a powder with mortar and pestle under liquid N_2 in the presence of polyvinylpolypyrolidone (70 mg). Extraction buffer[31] (1 mL, 0.5 M NaCl, adjusted to pH 8.3 by addition of NaOH) was added together with 10 mM DTT and Complete Protease Inhibitor Cocktail tablets (Roche Diagnostics NZ, Auckland, NZ). Samples were left on ice for 1 h and then cleared by centrifugation (16 000*g*, 4 °C, 10 min). Protein concentration was determined by Bradford staining[40] using bovine serum albumin (BSA) as standard. Protein samples (4 μg) were loaded onto 4−12% NuPAGE gels (Invitrogen, Carlsbad, CA) and run in MOPS buffer for 90 min at 150 V using a Mini-PROTEAN 3 electrophoresis system (Bio-Rad, Hercules, CA). Loading buffer[41] contained 100 mM Bis-Tris pH 6.6, 4 mM DTT with 19% (w/v) glycerol and 0.02% Brilliant Blue G. Gels were stained with modified colloidal Coomassie G250 stain[42] to visualize proteins.

Proteins were electroblotted onto polyvinyldifluoride membrane (Immobilon Transfer Membrane, pore size 0.45 μm, Millipore, Bedford, MA) in BSN transfer buffer[43] (48 mM Tris, 39 mM glycine, 0.1% (w/v) SDS, 20% methanol, pH 9.2). A semidry transfer cell (Bio-Rad) was used for transfer at 5 V for 5 h. Membranes were blocked with TBS buffer (10 mM Tris base, 150 mM NaCl, pH 8) plus 0.1% (v/v) Tween20 and 5% nonfat milk powder (Anchor, Auckland, NZ) at room temperature for at least 2 h. After washing the membrane extensively with TBS buffer + 0.05% Tween20 (TBST), proteins were immunodetected with kiwifruit antibodies for actinidin, kiwellin, or TLP (1:500 (w/v), diluted in TBS buffer containing milk powder). After extensive washing with TBST, membranes were incubated with an antirabbit secondary antibody (Qdot 655 goat $F(ab')_2$ antirabbit IgG conjugate (H+L), Invitrogen) in TBST at 1:1000 (w/v) together with an alkaline phosphatase conjugated antirabbit secondary antibody (Sigma-Aldrich at 1:1000 w/v). Binding was visualized using the Typhoon 9400 Variable Mode Imager (excitation 457 nm, emission 520 nm, band pass 40 nm, photomultiplier tube at 500 V). Protein levels were calculated using the area of each individual band (set manually) multiplied by the average intensity of each band quantified using ImageQuant TL 7.0 software (Pharmacia, Uppsala, Sweden).

Sequence Identification. The PFR *Actinidia* EST database[44] was BLAST searched for sequences with homology to published actinidin (GenBank accession number EF530131), kiwellin (P84527), and TLP (P81370) sequences. Full-length ESTs from fruit libraries of *A. deliciosa*, *A. chinensis*, *A. arguta*, and *A. eriantha* were identified (BLAST score < exp^{-60}) and selected for complete sequencing. Sequence alignments were constructed using ClustalX (version 1.8).

LC-MS Analysis of Peptides. Bands for actinidin, kiwellin, and TLP were excised for LC-MS analysis from Coomassie-stained 1-D SDS PAGE gels, and the protein was digested with 0.65 μg of trypsin (modified sequencing grade, Roche, Mannheim, Germany) in 0.2 M ammonium bicarbonate and 4.3% acetonitrile. Tryptic peptides were separated and analyzed using an Ettan multidimensional liquid chromatograph (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) coupled to a LTQ linear ion trap mass spectrometer with a nanospray electrospray ionization interface (Thermo Fisher Scientific Inc., San

Figure 1. Fifteen varieties of kiwifruit analyzed in this study. (Top left panel) Different tissue zones present in kiwifruit: outer pericarp, inner pericarp (seed, locules, septum), core (columella), and peel (exocarp). Each subsequent panel shows an external view of a representative whole mature fruit (right) and its corresponding transverse midsection (left). Panels are labeled sequentially from left to right: *A. deliciosa* 'Hayward', 'Bruno', and 'Qinmei'; *A. eriantha* GPA, GPB, PPA, and PPB; *A. chinensis* 'Hort16A', 'Lushan-xiang', 'Jin Feng', and 'Hongyang'; *A. arguta* 'Hortgem Wha', 'Hortgem Tahi', 'Hortgem Rua', and 'Hortgem Toru'. Scale in bottom right corner (in cm).

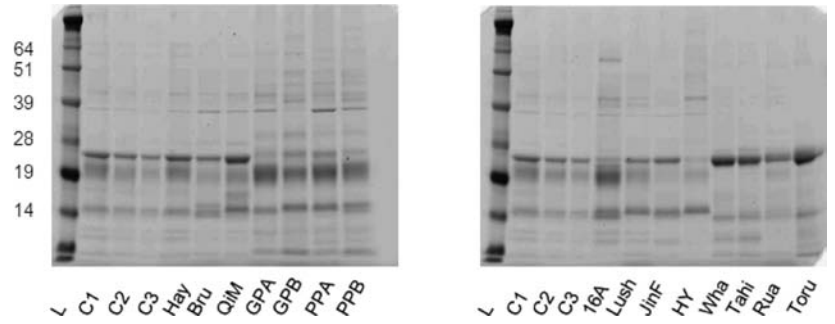

Figure 2. Representative 1-D SDS PAGE of protein extracts (4 μg) from whole fruit of 15 varieties of kiwifruit. L = SeeBluePlus2 prestained ladder (Invitrogen); sizes in kDa. C1−C3 = *A. deliciosa* 'Hayward' at 1, 0.5, and 0.3 dilution; *A. deliciosa* Hay = 'Hayward', Bru = 'Bruno', QiM = 'Qinmei'; *A. eriantha*, GPA, GPB, PPA, PPB; *A. chinensis* 16A = 'Hort16A', Lush = 'Lushan-xiang', JinF = 'Jin Feng', HY = 'Hongyang'; *A. arguta* Wha = 'Hortgem Wha', Tahi = 'Hortgem Tahi', Toru = 'Hortgem Toru', and Rua = 'Hortgem Rua'.

Jose, CA) as described in Nieuwenhuizen et al.[27] MS data were acquired using a top three experiment in data-dependent mode with dynamic exclusion enabled. This method isolates and fragments the most intense ion in the full scan to give MS/MS data and then isolates and fragments the second most intense ion, followed by the third most intense ion before performing another full scan. MS/MS data were analyzed using TurboSEQUEST protein identification software (Thermo Fisher Scientific Inc., San Jose, CA).

Actinidin Activity Measurements. Cysteine protease (CP) activity was measured using fluorescent substrates Z-FRAMC (benzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin), H-D-Ala-Leu-Lys-AMC, or Bz-Arg-AMC either with or without addition E-64 (a potent CP inhibitor). All substrates were obtained from Bachem Feinchemikalien AG, Bubendorf, Switzerland. Frozen tissues (1−2 g) were ground to a powder with mortar and pestle under liquid N_2 and then vortexed in the presence of two volumes of extraction buffer (1% glycerol, 100 mM Tris pH 8.5, 0.5% PVP-25, 1% PVPP, 0.095% $Na_2S_2O_5$, 5 mM DTT, 20 mM L-cysteine). After incubation for 30 min on ice, the extract was centrifuged for 5 min (5000*g*, 4 °C). The supernatant was transferred to a new tube and centrifuged for 10 min (14 000*g*, 4 °C). Extracts were frozen at −80 °C until required.

Activity assays were performed in 96-well white fluorescent microtiter plates (Nunc, Roskilde, Denmark) essentially as described in Rassam and Laing.[45] Protein extract (10 μL) was mixed with 100 μL of MOPS buffer (100 mM, pH 7) with or without E64 (25 μM) and incubated for 10 min. Appropriate substrate (10 μL, 2.5 mM in DMF) was then added, and the rate of released fluorescence was measured using a Victor 1420 multilabel counter (Perkin-Elmer Life Science, Turku, Finland) using excitation and emission wavelengths of 355 and 460 nm, respectively. Control reactions containing extraction buffer alone or boiled protein extract were used as blanks.

Protein Modeling. Three-dimensional structure predictions were carried out using SWISSMODEL,[46] available on the Expasy Web site (http://www.expasy.org). The program was run using project mode, and the template selected was the crystal structure of the *A. deliciosa* actinidin-E-64 complex[47] (Protein Data Bank code 1AEC). Template

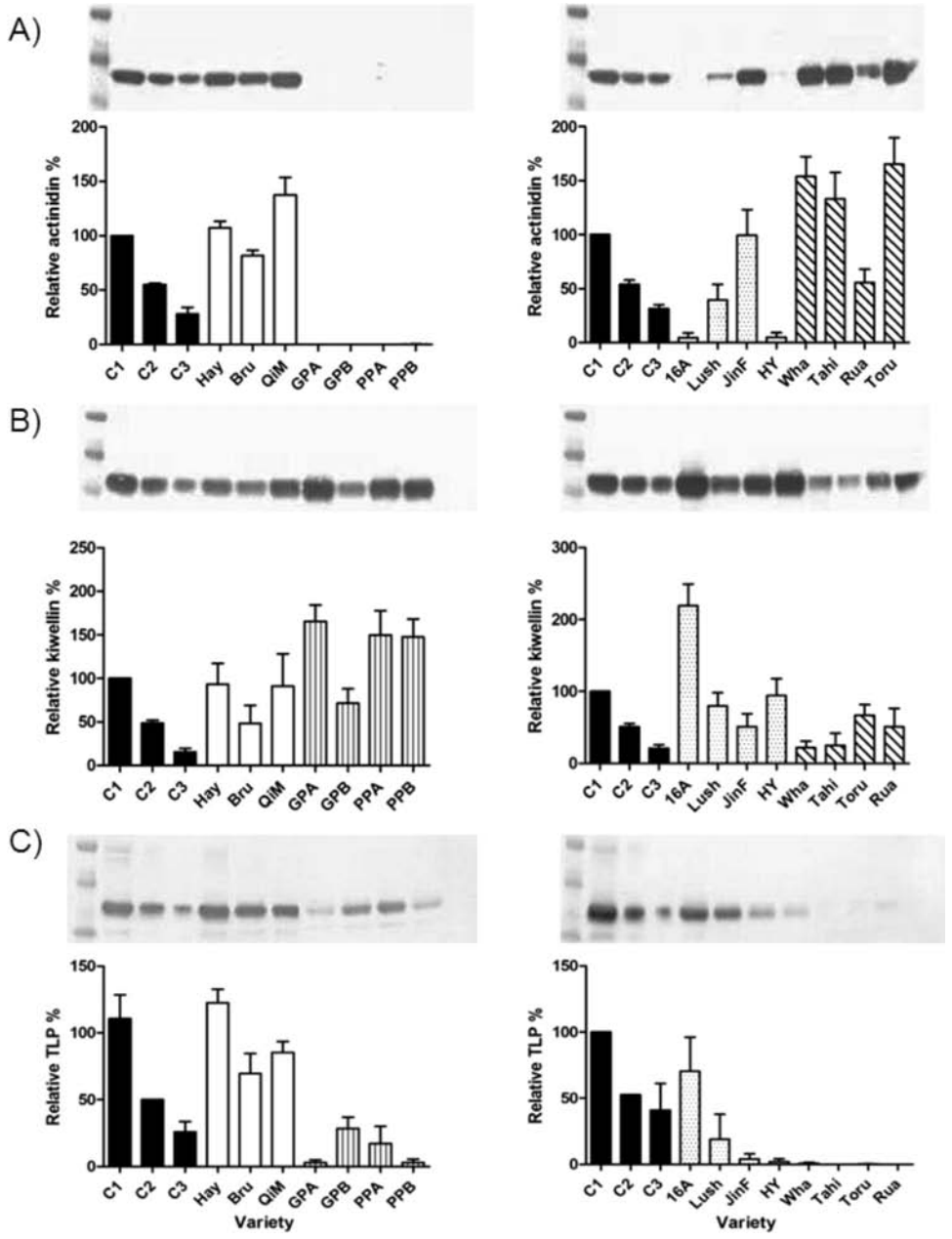

Figure 3. Western analysis and relative level of proteins using polyclonal antibodies to (A) actinidin, (B) kiwellin, and (C) thaumatin-like protein (TLP) from 15 varieties of kiwifruit. C1−C3 = *A. deliciosa* 'Hayward' at 1, 0.5, and 0.3 dilution; *A. deliciosa* Hay = 'Hayward', Bru = 'Bruno', QiM = 'Qinmei'; *A. eriantha*, GPA, GPB, PPA, PPB; *A. chinensis* 16A = 'Hort16A', Lush = 'Lushan-xiang', JinF = 'Jin Feng', HY = 'Hongyang'; *A. arguta* Wha = 'Hortgem Wha', Tahi = 'Hortgem Tahi', Toru = 'Hortgem Toru', and Rua = 'Hortgem Rua'. Error bars are SEM, $n = 3$. Three size markers on each western are 39, 28, and 19 kDa (from top to bottom). Relative levels were corrected for total protein concentration in each sample by Bradford staining[40] using bovine serum albumin (BSA) as standard. Level in the *A. deliciosa* 'Hayward' C1 sample was set at 100%.

and target sequences were 91.8% identical and 96.4% similar. The semirefined model was then sent to the SWISS-MODEL server for final refinement. Images were generated in the molecular graphics and modeling package Pymol (http://www.pymol.org/).

RESULTS AND DISCUSSION

Western Analysis of Actinidin, Kiwellin, and Thaumatin-Like Proteins. The relative level of three major allergens (actinidin, kiwellin, TLP) was characterized in the ripe fruit of 15 varieties of kiwifruit. These varieties were chosen as being representative of the four major species of *Actinidia* that are currently in worldwide cultivation. All varieties, except those from *A. eriantha*, are commercially available. Figure 1 shows representative whole and transverse sections of each fruit used in this study. Total proteins were extracted from whole eating-ripe fruit tissue including core, inner pericarp (containing both locules and seed), outer pericarp, and peel (Figure 1, top left). Although the peel of *A. deliciosa* and *A. chinensis* cultivars is typically not eaten (and the peel of *A. eriantha* is highly unpalatable due to its hairiness), peel tissue was included in all extractions as *A. arguta* fruit are eaten whole.

Three protocols were initially tested for extraction of total proteins to find a rapid, reproducible method that could be used on all 15 varieties: hot SDS[48] (extraction buffer, 8% SDS w/v, 24% glycerol v/v, 100 mM Tris HCl pH 8.6, 4% 2-

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Table 1. Fragments Obtained by LC-MS Analysis of Bands for Actinidin, Kiwellin, and Thaumatin-Like Protein (TLP) from *A. deliciosa* 'Hayward', *A. chinensis* 'Hort16A', *A. eriantha* GPA, and *A. arguta* 'Hortgem Tahi'[a]

	actinidin	kiwellin	TLP
Hay	TVGAVVGVK, 830	NVGVVDITWSM[b]A, 1308	TGC[c]NFDGAGR, 1055
	M[b]PSYPVK, 838	IVALSTGWYNGGSR, 1482	C[c]PDAYSYPK, 1101
	SAGAVVDIK, 860	C[c]NDDPQVGTHIC[c]R, 1573	GQNWIINPGAGTK, 1357
	GLM[b]TDAFK, 899	NNIVDGSNAVWSALGLDK, 1874	DRC[c]PDAYSYPK, 1372
	YVTIDSYK, 989	SHPTYDC[c]SPPVTSSTPAK, 1933	CTADINGQCPNELR, 1649
	GM[b]DYWIVK, 1028	LTNNDFSEGGDGGGPSEC[c]DESYHSNNER, 3046	DDQTSTFTC[c]PAGTNYK, 1807
	AVAYQPVSVGVESEGGK, 1677		GATFNIINNCPFTVWAAAVPGGGK, 2407
	NSWDTTWGEEGYM[b]R, 1749		
	KAVAYQPVSVGVESEGGK, 1806		
	NVGGAGTC[c]GIATM[b]PSYPVK, 1897		
	IVTGVLISLSEQELIDC[c]GR, 2103		
16A	TVGAVVGVK, 830	ITASNGKSVSAK, 1163	TGC[c]NFDGAGR, 1055
	M[b]PSYPVK, 838	NVGVVDITWSM[b]A, 1308	GQNWIINPGAGTK, 1357
	GLM[b]TDAFK, 899	IVALSTGWYNGGSR, 1482	GATFNIINNCPFTVWAAAVPGGGK, 2407
	YVTIDSYK, 989	NNIVDGSNAVWSALGLDK, 1874	
	GM[b]DYWIVK, 1028	SHPTYDC[c]SPPVTSSTPAK, 1933	
	NVPSNNEM[b]ALK, 1233	LTNNDFSEGGDGGGPSEC[c]DESYHSNNER, 3046	
	NSWGTNWGESGYIR, 1628	NNIVDGSNAVWSALGLDKNVGVVDITWSM[b]A, 3164	
	AVAYQPVSVGVESEGGK, 1678		
	IVTGNLISLSEQELVDC[c]GR, 2104		
	FIINNGGINTENNYPYTAK, 2144		
	YVTIDSYKNVPSNNEM[b]ALK, 2203		
GPA	no fragments	ITASNGKSVSAK, 1163	TGCNFDGAGR, 1055
		NVGVVDITWSM[b]A, 1308	GATFNIINNCPFTVWAAAVPGGGK, 2407
		IVALSTGWYNGGSR, 1482	
		NNIVDGSNAVWSALGLDK, 1874	
		SYPTYDCSPPVTSSTPAK, 1959	
		LTNNDFSEGGDGGGPSECDER, 2214	
		NNIVDGSNAVWSALGLDKNVGVVDITWSM[b]A, 3164	
Tahi	SAGAVVDIK, 860	NVGVVDITWSM[b]A, 1308	no fragments
	GLDYWIVK, 994	IVALSTGWYNGGSR, 1482	
	NVGGAGTC[c]GIATK, 1206	NNIVDGSNAVWSALGLDK, 1874	
	NSWDTTWGEEGYIR, 1715		
	NVGGAGTCGIATMPSYPVK, 1897		
	IVTGNLISLSEQELVDCGR, 2104		
	IVTGDLISLSEQELVDC[c]GR, 2105		
	SQGQCGSCWAFSAIATVEGINK, 2259		

[a]The MH^+ ion for each fragment is given. [b]Oxidized methionine residues. [c]Alkylated cysteine residues.

mercaptoethanol v/v), a protocol based on Tamburrini et al. [31] (high salt extraction buffer, 0.5 M NaCl, 10 mM DTT), and a protocol for extraction of proteins for 2-D electrophoresis[49] (lysis buffer, 7 M urea, 2 M thiourea, 40 mM Tris, 75 mM DTT, 4% CHAPS v/v). All three protocols worked efficiently; however, in our hands, the high salt extraction protocol from Tamburrini et al.[31] was the easiest to use and gave the most reproducible extractions, with total protein varying by <25% across all varieties tested. Proteins were extracted in the presence of high levels of DTT to ensure complete reduction of proteins. Figure 2 shows that the proteins extracted from each species were quite similar with major bands around 24, 22, and 15 kDa present in most species. Although all extractions were performed in the presence of a cocktail of protease inhibitors to prevent protease digestion (especially by actinidin that is known to have CP activity), the predominance of proteins were extracted in the lower molecular weight range.

Polyclonal antibodies raised to recombinant proteins of actinidin, kiwellin, and native TLP were used to immunodetect proteins across the 15 kiwifruit varieties tested. For each analysis at least three extractions were performed, and the results were expressed relative to the signal obtained from a dilution series of *A. deliciosa* 'Hayward' extract run on each gel. Variation within the replicate extractions from the same variety was less than that observed between species and between genotypes within the same species (Figure 3A–C). Immunodetection of proteins in different tissue zones of selected samples showed the same variation as whole fruit samples (Figure S1A, Supporting Information).

For actinidin, a band at ~24 kDa was observed (Figure 3A). Actinidin was present in all three *A. deliciosa* varieties tested but was not detected in any of the four *A. eriantha* varieties. In *A. arguta*, three of the varieties ('Hortgem Wha', 'Hortgem Tahi', and 'Hortgem Toru') contained higher levels of actinidin than 'Hayward' while 'Hortgem Rua' was intermediate at ~50%. The greatest in species variation occurred within the *A. chinensis* varieties, with 'Hort16A' and 'Hongyang' showing trace amounts of actinidin protein, 'Jin Feng' levels comparable to those of 'Hayward', and 'Lushan-xiang' having an intermediate level (40%). The low levels of actinidin in 'Hort16A' are

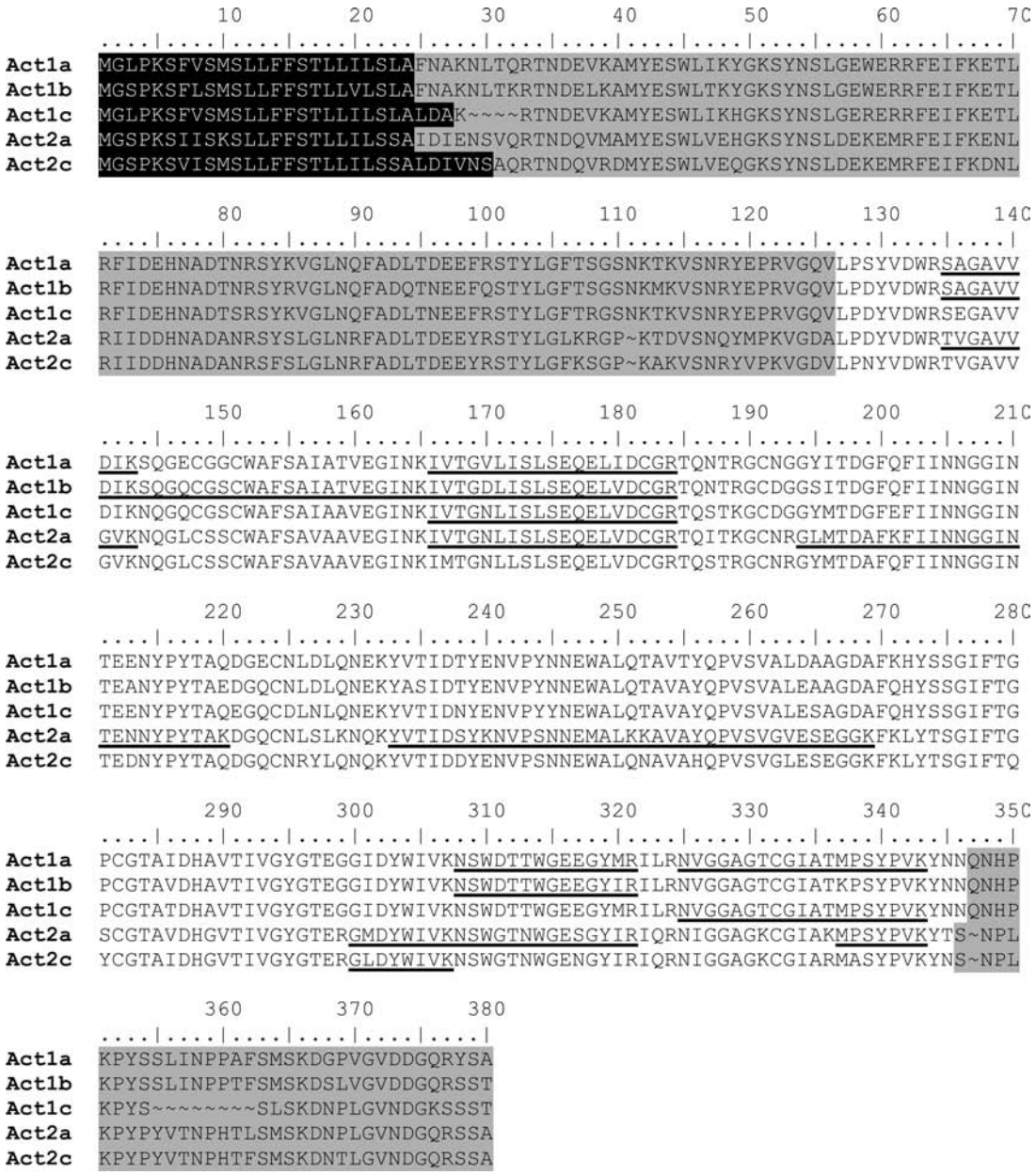

Figure 4. Amino acid alignment of predicted full-length actinidin (Act) protein sequences reported by Nieuwenhuizen et al.[27] Black shading indicates the cleaved signal peptide. Gray shading indicates the N-terminal and C-terminal propeptide sequences that are cleaved to produce the active cysteine protease enzyme. '~' indicates gaps inserted for optimal alignment. Underlining highlights the position of fragments identified by LC-MS from actinidin bands (Table 1, column 1). LC-MS fragments from *A. deliciosa* 'Hayward' and *A. chinensis* 'Hort16A' are aligned with *Act1a* and *Act2a* (GenBank accession numbers EF530131 and EF530134), *A. arguta* 'Hortgem Tahi' fragments are aligned with *Act1b*, *Act1c*, and *Act2c* (EF530132, EF530133, EF530136).

consistent with previous reports[25,27] and have been shown to result from inactivation of both alleles encoding the major acidic form of actinidin at the *ACT1A* locus.[29]

For kiwellin a band at ~20 kDa was observed in all varieties examined (Figure 3B). The three *A. deliciosa* varieties contained similar levels of kiwellin. The levels in three of the four *A. eriantha* varieties were higher than 'Hayward', while all *A. arguta* samples were lower than 'Hayward'. In *A. chinensis*, 'Lushanxiang', 'Jin Feng', and 'Hongyang' contained similar levels to 'Hayward', while 'Hort16A' contained significantly higher quantities. There was some suggestion of an inverse relationship between levels of kiwellin and actinidin (cf. *A. eriantha* varieties, 'Hort16A' and 'Hongyang' with low levels of actinidin and high levels of kiwellin v. *A. arguta* varieties with high actinidin and low kiwellin levels).

TLP was observed as a band of ~22 kDa (Figure 3C). The total levels of TLP protein were typically lower than for actinidin or kiwellin, as evidenced by the lower intensity of banding on the immunoblots. TLP levels were highest in 'Hayward' and the two other *A. deliciosa* varieties ('Bruno' and 'Qinmei'). Of the other varieties, only *A. chinensis* 'Hort16A' showed TLP levels comparable to 'Hayward'. TLP levels in all four *A. arguta* varieties, *A. chinensis* 'Jin Feng' and 'Hongyang', and *A. eriantha* GPA and PPB were very low.

The cross reactivity of the polyclonal antibodies was tested against protein extracts from a range of other fruit—pear,

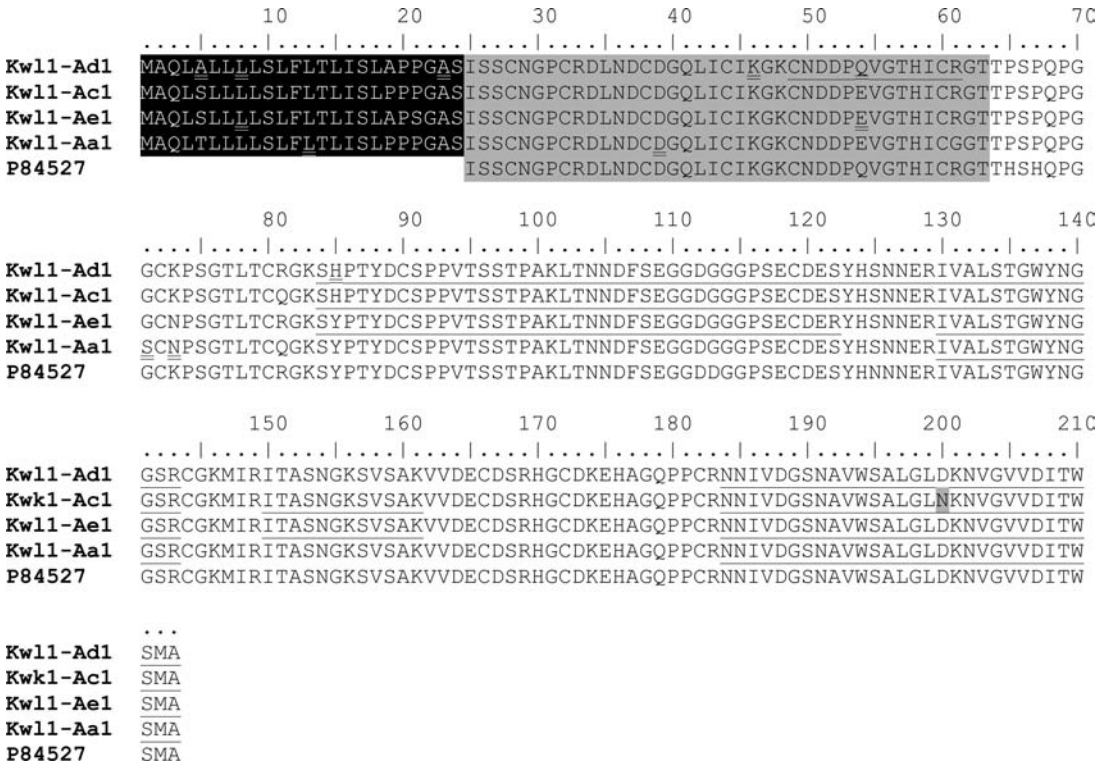

Figure 5. Amino acid alignment of predicted full-length kiwellin (Kwl) sequences obtained from the PFR *Actinidia* EST database and the published kiwellin sequence (GenBank accession number P84527). *Kwl1* sequences were obtained from *A. deliciosa* (Ad, JX905290), *A. chinensis* (Ac, JX905294), *A. eriantha* (Ae, JX905295), and *A. arguta* (Aa, JX905298). Double underlining indicates that polymorphisms were identified in cDNA sequences at these residues (see Table S1, Supporting Information, for these individual polymorphic sequences). Black shading indicates the predicted signal peptide. Gray shading indicates the 4 kDa kissper peptide cleaved from kiwellin to produce the 16 kDa KiTH protein.[26] Single underlining highlights fragments identified by LC-MS analysis from kiwellin bands (Table 1, column 2). LC-MS fragments from each species are aligned with the *Kwl1* sequence from that respective species. Single gray highlighted residue was in conflict between the *Kwl1-Ac1* sequence and the LC-MS fragments obtained from *A. chinensis* 'Hort16A'.

grape, orange, banana, tomato, plum, avocado, and apple (Figure S1B, Supporting Information). No bands between 19 and 39 kDa were observed in any species using the actinidin polyclonal antibody, while only weak nonspecific bands significantly larger than expected were seen using the kiwellin antibody. The TLP antibody detected a strong band of the expected 22 kDa size in banana and avocado and a weak band in grape. Overall, the epitopes identified by the three antibodies appear to exist across a range of *Actinidia* species, but these epitopes do not appear to be commonly found in other fruit (except for TLP).

The western results using polyclonal antibodies to recombinant actinidin and kiwellin proteins and native TLP indicate that there is significant variation in the relative levels of actinidin, kiwellin, and TLP proteins within individual kiwifruit species as well as between species. The results confirm the high actinidin levels found in *A. deliciosa* exemplified by the 'Hayward' cultivar and show the low level found in 'Hort16A' is not unique in *A. chinensis* to this cultivar. For kiwellin and TLP, variation in these key proteins is reported for the first time across a range of important kiwifruit species. The wide variation in protein levels we observed emphasizes the care that needs to be taken when generalizing about the allergenicity of a particular kiwifruit species and the importance of correct cultivar identification when reporting allergenicity studies (e.g., reports in which fruit are described as being from local markets do not allow easy verification of results; see Materials in refs 21 and 31).

LC-MS Analysis and Sequence Identification. To identify the major protein isoforms of actinidin, kiwellin, and TLP expressed in ripe kiwifruit, bands corresponding to each protein were cut out from 1-D SDS-PAGE gels, trypsin digested, and subjected to LC-MS analysis. Bands were analyzed from four varieties, namely, 'Hayward', 'Hort16A', GPA, and 'Hortgem Tahi', representative of the four *Actinidia* species in this study. LC-MS fragments were identified by BLAST searching the PFR *Actinidia* EST database.[44] Fragments with homology to actinidin, kiwellin, and TLP sequences are presented in Table 1.

For actinidin, fragments corresponding to two isoforms were identified in ripe 'Hayward' fruit (Table 1, column 1). Five fragments (31% coverage of mature enzyme) could be aligned to the sequence of the major acidic isoform ACT1A,[13,14,18,27] while seven fragments, (26% coverage) aligned to a basic isoform, ACT2A.[19,27] One fragment was common to both isoforms. Alignments are shown in Figure 4. Fragments of the basic ACT2A isoform were also detected in 'Hort16A' (11 fragments, 58% coverage). In *A. arguta* 'Hortgem Tahi', fragments aligned with two acidic isoforms ACT1b (4 fragments) and ACT1c (2 fragments) and the basic isoform ACT2c (1 fragment).

For kiwellin, very little variation in predicted protein sequences between *Actinidia* species was observed (Figure 5). The sequences showed >95% identity to each other and are likely to represent a single locus in each species. The predicted kiwellin sequence from 'Hayward' differed by five residues from

Figure 6. Amino acid alignment of predicted full-length thaumatin-like protein (TLP) sequences obtained from the PFR *Actinidia* EST database. TLP1, 3, and 4 sequences (GenBank accession numbers JX905282, JX905285, JX905287) were obtained from *A. deliciosa*, TLP2 was obtained from *A. chinensis* (JX905284). Double underlining indicates that polymorphisms were identified in cDNA sequences at these residues (see Table S1, Supporting Information, for these individual polymorphic sequences). Black shading indicates the predicted signal peptide. Single underlining highlights fragments identified by LC-MS from TLP bands (Table 1, column 3). All LC-MS fragments from *A. deliciosa*, 'Hayward' *A. chinensis* 'Hort16A', and *A. eriantha* GPA are shown aligned against TLP1. '~' indicate gaps inserted for optimal alignment.

the published kiwellin sequence that was obtained by direct protein sequencing of protein isolated from *A. chinensis* kiwifruit (no cultivar specified[31]). Interestingly, two of these residue differences (positions 65 and 67) were conserved in kiwellin sequences from all four *Actinidia* species used in this study and occurred within the predicted cleavage site for kiwellin to produce the 4 kDa kissper peptide and 16 kDa KiTH protein.[26] If these differences affected cleavage, it might explain why we did not observe the 16 kDa KiTH protein in western analysis in any of our samples. It would be interesting to test this hypothesis in vitro using the purified recombinant kiwellin protein produced in this study. LC-MS fragments for kiwellin showed little variation between species (Table 1, column 2), and all fragments could be aligned to the KWL1 isoform. Six LC-MS fragments (54% coverage) were obtained for 'Hayward' including one that aligned with the kissper peptide and five with the KiTH protein (Figure 5).

For TLP, four divergent predicted protein sequences were obtained in the PFR *Actinidia* EST database (Figure 6). These sequences showed 60−74% amino acid identity to each other. All LC-MS fragments corresponded to TLP1, an isoform previously reported.[21,50] In general, there were relatively few LC-MS fragments obtained for TLP across all four species with a maximum of seven fragments in *A. deliciosa* 'Hayward' (44% coverage of mature protein), only three from *A. chinensis* 'Hort16A' and two from *A. eriantha* GPA. No fragments were obtained in *A. arguta* 'Hortgem Tahi', consistent with the lack of immunodetectable protein (Table 1, column 3).

These results suggest that single major isoforms of actinidin, kiwellin, and TLP are expressed in the ripe fruit from the four *Actinidia* species investigated. Although the LC-MS methodology employed is not fully quantitative and can be biased by hydrophobicity and the ability of a protein to be digested by trypsin and generate peptides of detectable sequence length, it is likely that ACT1a, KWL1, and TLP1 are the major isoforms expressed in these fruit and primarily responsible for the bands immunodetected in the western analysis (Figure 3). Data do not indicate whether the most abundant isoforms are also the most allergenic to consumers. Allergenicity can be highly specific to individual protein isoforms, e.g., Breiteneder et al.[51] Therefore, it is possible that a highly specific variant (e.g., representing one of the polymorphic cDNA sequences, Table S1, Supporting Information) or a less abundant but more divergent isoform may represent the most allergenic protein isoform of actinidin, kiwellin, and TLP in kiwifruit.

Actinidin Activity in 15 Kiwifruit Varieties. As a surrogate for protein/allergen levels, the cysteine protease (CP) activity of actinidin was measured using a fluorometric assay with Z-FRAMC as substrate (Figure 7A) in the presence and absence of E-64 (a potent CP inhibitor). CP activity was low in all varieties in which low levels of actinidin protein were immunodetected including the four *A. eriantha* varieties, *A. chinensis* 'Hort16A', and 'Hongyang'. In the three *A. deliciosa* varieties and the *A. chinensis* varieties 'Lushang-xiang' and 'Jin Feng', CP activity correlated with the levels of actinidin protein. In *A. arguta*, the situation was more complex. 'Hortgem Toru', with high levels of actinidin protein, also showed high CP activity as expected. However, 'Hortgem Wha', 'Hortgem Tahi', and 'Hortgem Rua' all contained high levels of immunodetectable actinidin protein but showed only low levels of CP activity. CP activity was abolished in the presence of the CP-specific inhibitor E-64 as expected. CP assays using the alternative

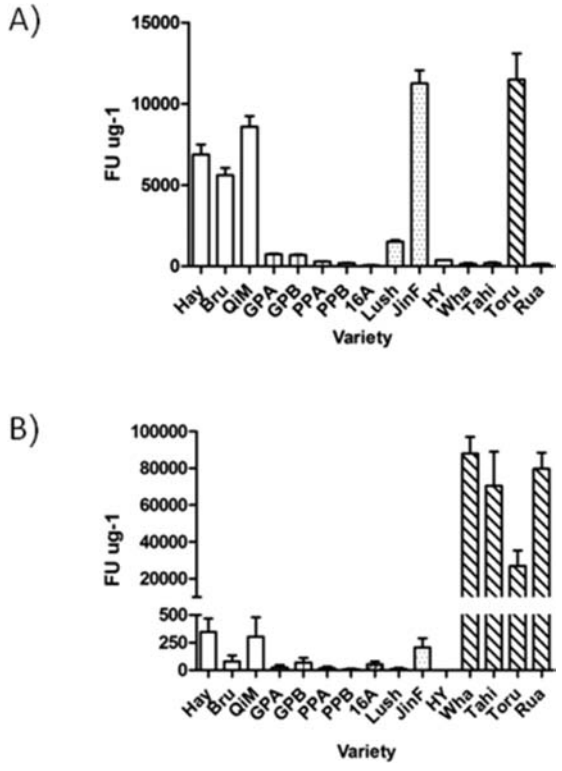

Figure 7. Cysteine protease activity in protein extracts from 15 varieties of kiwifruit measured using a fluorometric assay with (A) Z-FRAMC and (B) Bz-Arg-AMC as substrates. *A. deliciosa* Hay = 'Hayward', Bru = 'Bruno', QiM = 'Qinmei'; *A. eriantha*, GPA, GPB, PPA, PPB; *A. chinensis* 16A = 'Hort16A', Lush = 'Lushan-xiang', JinF = 'Jin Feng', HY = 'Hongyang'; *A. arguta* Wha = 'Hortgem Wha', Tahi = 'Hortgem Tahi', Toru = 'Hortgem Toru', and Rua = 'Hortgem Rua'. All assays were performed in quadruplicate from three different tissue extractions. Activity is given as the rate of released fluorescence per milligram of total protein. Data were corrected for total protein concentration in each sample by Bradford staining[40] using bovine serum albumin (BSA) as standard. Error bars are SEM.

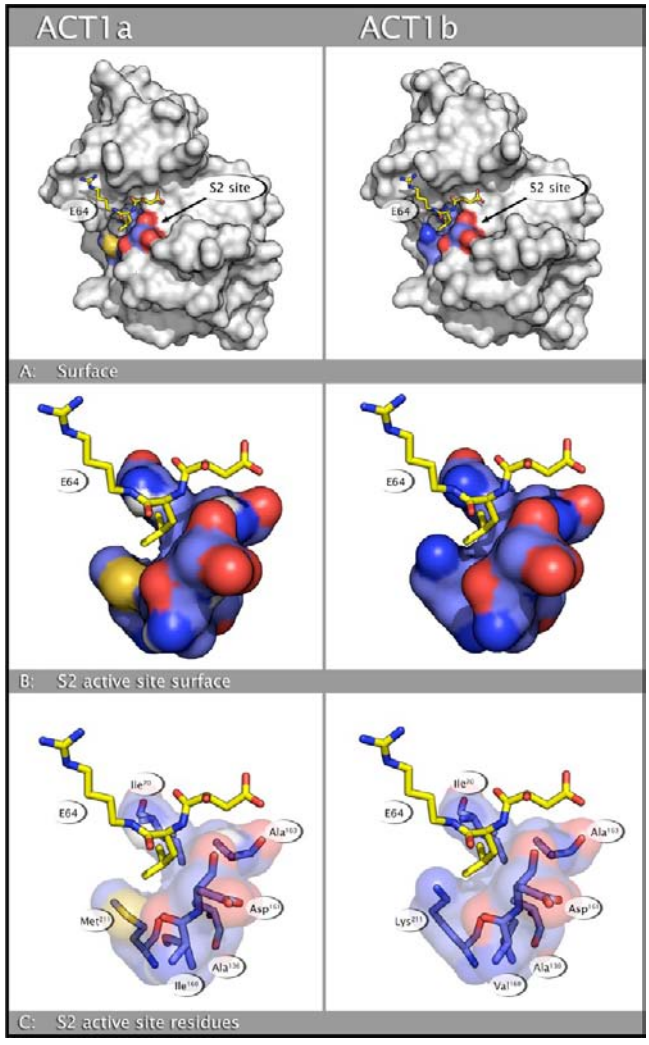

Figure 8. S2 binding pocket structural variation between ACT1a and ACT1b. (A) Surface representation of the ACT1a crystal structure[47] and ACT1b homology model showing the S2 hydrophobic binding pocket (colored region) and the E-64 inhibitor (yellow). (B) Close-up surface representation of the S2 binding pockets in ACT1a and ACT1b. (C) Underlying S2 binding pocket residues for ACT1a and ACT1b. $Met^{211} \rightarrow Lys^{211}$ difference potentially explains the inability of ACT1b to bind substrates with aromatic substituents due to a shortening of the S2 binding pocket.

fluorescent substrate H-D-Ala-Leu-Lys-AMC confirmed the results with Z-FRAMC and the low activity of 'Hortgem Wha', 'Hortgem Tahi', and 'Hortgem Rua' (data not shown). However, when Bz-Arg-AMC was used as the substrate (Figure 7B), CP activity was observed in the four *A. arguta* varieties, and activity was very low or absent in all other varieties. CP activity was again abolished in the presence of the CP inhibitor E-64 in all cases.

These results indicate that using the CP activity assay as a surrogate for protein/allergen levels requires an understanding of the specificity of the actinidin enzyme. Structural comparisons of a protein homology model for ACT1b (the isoform likely to be found at highest level in *A. arguta* 'Hortgem Tahi'[27]) with the crystal structure of ACT1a (the isoform found at highest levels in *A. deliciosa* 'Hayward', PDB code 2AEC) indicated that an S2 subsite lysine residue (Lys^{211}) residue might account for the observed CP activity differences (Figure 8). Structural differences in the hydrophobic S2 subsite (which largely defines substrate specificity in CPs[52−56]) between 'Hayward' ACT1a and closely related papain enzymes have already established that substitution of a serine residue (Ser^{205}) located at the bottom of the S2 binding pocket in papain with Met^{211} in ACT1a causes a 10- to 100-fold decrease in ACT1a catalytic efficiency for substrates with aromatic substituents in the P2 position.[57,58] This catalytic effect is explained by a 'shortening' of the hydrophobic pocket due to the more extended Met side chain. It is reasonable to assume therefore that substitution of the S2Met residue in ACT1a by Lys^{211} in ACT1b could prevent aromatic substituent binding due to an additional shortening of the hydrophobic pocket. The assumption is supported by the selective activity of ACT1b with Bz-ARG-AMC (Figure 7B).

Our results indicate that significant variation exists for actinidin, kiwellin, and TLP in ripe kiwifruit and that care needs to be taken when extrapolating allergenicity results both within and between *Actinidia* species. This observation is consistent with results reported by Le et al.,[59] who showed differences in the allergenicity of six different kiwifruit varieties using prick-to-prick testing, open food challenges, and ELISA. The data provided here on specific isoforms expressed in ripe fruit will allow recombinant proteins to be made and tested against

allergenic patient sera.[16,17] This information can then be supplied to clinicians and patients on the allergenic potential of individual kiwifruit cultivars. Finally, we show that using enzyme activity assays as a surrogate for protein/allergen levels across divergent species requires an understanding of enzyme substrate specificity.

■ ASSOCIATED CONTENT

Supporting Information

Actinidin, kiwellin, and thaumatin-like proteins (TLP) in different kiwifruit tissue zones and in non-*Actinidia* species; sequences of kiwellin and thaumatin-like proteins (TLP). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

EST, expressed sequence tag; LC-MS, liquid chromatography–mass spectrometry; TLP, thaumatin-like protein

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