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Identification and molecular characterization of allergenic nsLTP from durum wheat (*Triticum turgidum*)

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Abstract

Background: Common wheat (*Triticum aestivum*) and durum wheat (*Triticum turgidum*) are both involved in baker's asthma (BA) and food allergy (FA) including WDEIA. However, allergens in durum wheat have not been described, and the over-expression of *T. turgidum* nsLTPs is considered to increase resistance to phytopathogens.

Objective: To identify and assess the allergenicity of nsLTP from *T. turgidum*.

Methods: Recombinant *T. turgidum* nsLTP Tri tu 14 was generated and tested for structural integrity (CD-spectroscopy) and purity (SDS-PAGE). Thirty-two wheat allergic patients were enrolled: 20 Spanish patients (BA) with positive bronchial challenge to wheat flour, and 12

Italian patients (wheat FA/WDEIA) with positive DBPCFC/OFC to pasta. IgE values to wheat, Tri tu 14, Tri a 14 (*T. aestivum*) and Pru p 3 (*P. persica*) were determined by ImmunoCAP testing. Allergenic potency (*in vitro* mediator release) and IgE cross-reactivity were investigated.

Results: Tri tu 14 was found to share 49% and 52% amino acid identity with Tri a 14 and Pru p 3, respectively. Among 25 Tri a 14 CAP positive sera, 23 (92%) were reactive to wheat extract, 22 (88%) to Tri tu 14 and 20 (80%) to Pru p 3. The correlation between Tri a 14 and Tri tu 14 specific IgE levels was r=0.97 (BA) and r=0.93 (FA/WDEIA), respectively. FA/WDEIA patients showed higher specific IgE values to Tri tu 14 and Pru p 3 than BA patients. Tri tu 14 displayed allergenic activity by mediator release from effector cells and IgE cross-reactivity with Pru p 3. The degree of IgE cross-reactivity between the two wheat nsLTPs varied between individual patients.

Conclusions & Clinical Relevance: Sensitization to Tri tu 14 likely appears to be more important in wheat FA/WDEIA than in baker´s asthma. Over-expression of Tri tu 14 in wheat would represent a risk for patients with nsLTP-mediated food allergy.

Introduction

Wheat is considered as a staple food in the diet worldwide and a known trigger of IgE-mediated allergies [1]. The most consumed wheat cultivars are common wheat (*Triticum aestivum* L.*)*, a main ingredient in bread and durum wheat (*Triticum turgidum ssp*. *durum*) which is preferentially used for pasta and semolina. Allergic reactions to wheat can be elicited either by wheat ingestion causing food allergy and wheat-dependent exercise-induced asthma (WDEIA), or by inhalation of wheat flour causing baker´s asthma [1].

In general, common wheat is a source of numerous allergens which can be classified into two groups: the first group comprising the salt insoluble fraction, including gliadin and gluten which constitutes approximately 80% of wheat protein content [2, 3]. Wheat proteins of this group have been described as allergens involved in celiac disease and in WDEIA [4]. The second group is represented by the albumin/globulin soluble fraction and comprises 15%-20% of total wheat proteins, including allergenic α-amylase/trypsin inhibitor subunit as well as the non-specific lipid transfer proteins (nsLTPs) [5]. Both allergens are implicated in baker's asthma [4, 6], and in some cases of WDEIA [7].

In line with this, nsLTPs which belong to the family of pathogenesis-related proteins (PR14) have been identified as major allergens in plant-derived foods, with a highest prevalence of sensitization in the Mediterranean area [8–10]. Several members of this protein family, particularly nsLTPs from *Rosaceae* fruits (such as peach nsLTP Pru p 3), have been proposed as a model of true food allergens based on their high resistance to both digestive proteolysis and heat treatment [9–13]. The characteristics of the nsLTPs panallergen family are displayed also by the wheat flour nsLTP (Tri a 14) which was reported to be an important food allergen in *T. aestivum*-derived food products [5, 14, 15]. Palacin and co-workers [16] have characterized Tri a 14 as a major inhalant allergen associated with baker's asthma in Spain. Specific IgE to Tri a 14 was detected in 60% of sera from 40 Spanish patients with baker's asthma. In contrast, Sander and co-workers [17] reported Tri a 14 as a minor allergen in patients with baker´s asthma, with sensitization to Tri a 14.01 being more prominent than sensitization to Tri a 14.02. They found 8% of German and Dutch bakers to be sensitized to Tri a 14.01 whereas only 3% were sensitized to Tri a 14.02. In comparison to this 21% of Spanish bakers were sensitized to Tri a 14.01 but only 13% to Tri a 14.02.

Non-specific LTPs are involved in various physiological processes including the response to phytopathogens in plant defense reactions [18]. In line with this, research activities have been initiated to upregulate nsLTPs in durum wheat to enhance tolerance to abiotic and biotic stress [19]. However, no data describing the potential allergens in durum wheat are available until now. Hence, the selective over-expression of nsLTPs in *T. turgidum* could be of a major risk for nsLTP allergic patients, especially for wheat allergic patients suffering from reactions to pasta [20].

The aim of the present study was the molecular cloning, characterization and allergenicity assessment of the nsLTP from durum wheat, and the comparison of IgE-reactivity, cross-reactivity and allergenicity with the major allergens Tri a 14 and Pru p 3. The study results suggest a role of Tri tu 14 in wheat food allergy and baker´s asthma.

Abbreviation

nsLTP: non-specific Lipid-Transfer Protein
WDEIA: Wheat-dependent exercise-induced asthma
K. pastoris: *Komagataella pastoris*
BA: Baker's asthma
FA: Food allergy
CD-spectroscopy: Circular dichroism spectroscopy

Material & Methods

Wheat allergic patients & patient´s sera

A total of 39 subjects were recruited for the study, comprising two patient groups: (1) 20 Spanish wheat allergic patients with baker´s asthma caused by wheat flour inhalation, and (2) 12 Italian patients with wheat food allergy (FA) including WDEIA. All patients were pre-

selected by sensitization to natural Tri a 14 from *Triticum aestivum* seeds by skin testing (for patients with baker´s asthma) or to recombinant Tri a 14.01 by ImmunoCAP testing (for wheat FA/WDEIA). Moreover, 7 Italian wheat nsLTPs sensitized (Tri a 14 ImmunoCAP positive) subjects but without any symptoms to ingestion of wheat were enrolled as a control group (**Tab. 1**). Manifestation of wheat allergy was verified as follows: (1) Patients with baker´s asthma underwent bronchial challenge tests (BCW) and skin prick tests (SPT). SPT was perfomed in accordance with the European Academy of Allergology and Clinical Immunology (EAACI) criteria with *T. aestivum extract* (1 ng/ml-10 µg/ml) and with purified nTri a 14 and nPru p 3 (100 pg/ml-1 µg/ml). The papule area was measured after 15 minutes and traced for posterior measurement by planimetry. A papule ≥19.62 mm^2 corresponding to a diameter of approximately 5 mm was considered as clearly positive [21]. Moreover, (2) WDEIA was confirmed by open challenge test using a standard protocol with 100 g of cooked pasta (*T. turgidum*) plus exercise [7], and (3) wheat FA was confirmed in all patients subjected to double-blind placebo-controlled food challenge (DBPCFC) [20]. Challenges were not performed in two wheat food allergic patients with history of severe anaphylaxis and one patient with associated baker´s asthma. Clinical studies were approved by the local ethical committees, Milano Area 3 ASST Grande Ospedale Metropolitano Niguarda Piazza Ospedale Maggiore (3252-052017), and Comité Etico de la Universidad Politécnica de Madreid' (BIO2017-84548-R).

Molecular cloning and purification of *T. turgidum* nsLTP, and recombinant Tri a 14 & Pru p 3

A full-length cDNA of *T. turgidum* nsLTP Tri tu 14 (TdLTP4, Gen-Bank accession no. JF799976) was cloned and sequenced as described elsewhere [19]. For recombinant expression in *K.*

pastoris (X33) a codon optimized synthetic gene (GeneArt Life Technologies, Regensburg, Germany) was cloned into the *EcoR*I and *Xba*I restriction sites of the yeast expression vector pPICZαA. Tri tu 14 was expressed as non-tagged protein and purified as described elsewhere [22] with slight modifications. Briefly, protein expression in 3 liter of *K. pastoris* culture was induced with 0.5% methanol at an OD_{600} = 1.0 for 5-6 days at 30°C. The supernatant was separated from the cell culture by centrifugation at 6,000 g for 20 min and subjected to cross-flow filtration (Ultrasette, 3 kDa, Pall, Dreieich, Germany). The filtrate (400 ml) was dialyzed against NaAc (50 mM, pH 5.0) and applied to cation exchange chromatography HiTrap SP/HP (GE Healthcare, Munich, Germany). Proteins were eluted applying a linear gradient from 0-1 M NaCl with NaAc (50 mM, pH 5.0). The eluted fractions containing the recombinant Tri tu 14 were collected and applied to size exclusion chromatography (Superdex 26/600, 75 pg, GE Healthcare, Munich, Germany) using 20 mM MOPS, 500 mM NaCl, pH 7.6 as running buffer. Natural Pru p 3 was purified as described elsewhere [23]. Recombinant Tri a 14.01 (Acc. No. CAH69187/AJ852536) was expressed as a secreted, hexahistidine-tagged protein in *K. pastoris*, using a codon-optimized synthetic gene, and purified from culture medium by immobilized metal ion affinity and anion exchange chromatography, followed by formulation in 20 mM MOPS, 0.15 M NaCl, pH 7.6.

Physicochemical characterization of allergens and protein extracts

Protein extracts from durum wheat (Tunisian cultivar Om Rabia3) and common wheat were prepared from whole grain and bran, respectively, using the method as described elsewhere [16] with some modifications. Proteins from ground seeds/bran (20 g each) were extracted with 100 mL Tris-HCl (100 mM), EDTA (10 mM, pH 4.5) for 1 hour at 4°C. The extract was

subjected to centrifugation (10,000 g for 30 minutes at 4°C), passed through a 0.45 µm filter and stored at 4°C for both ELISAs and histamine release assays.

Recombinant Tri tu 14, rTri a 14, nPru p 3 (2 µg/lane) and extracts of *T. turgidum* and *T. aestivum* (15 µg/lane) were subjected to SDS-PAGE (16%) under reducing conditions and GelCode Blue Safe Protein staining (Fisher Scientific, Nidderau, Germany). The protein concentration of wheat extracts was determined by Roti-Nanoquant 5× Bradford reagent (Carl Roth, Karlsruhe, Germany) while the protein content of purified proteins was determined by BCA protein assay kit (Fisher Scientific). The secondary structures of the purified recombinant proteins were analyzed by circular dichroism (CD) spectroscopy as described [22]. Modeling of the 3D-structure of Tri tu 14 was done using SWISS-MODEL (www.swissmodel.expasy.org) [24], and maize nsLTP (pdb: 1afh) as template. Overlay of structures were done using the PyMOL software (Molecular Graphics System, Version 2.0 Schrödinger, LLC). The identity of the protein was confirmed by in-gel digestion followed by LC/MS analysis according to [25] with slight modifications as specified in [26].

IgE-binding and cross-reactivity assays

Specific IgE values were determined by using experimental ImmunoCAP [27] for rTri tu 14 and commercially available ImmunoCAPs for rTri a 14.01 (f433), rPru p 3 (f420), and *T. aestivum* wheat (f4) (Thermo Fisher Scientific). Specific IgE levels were considered positive if ≥0.35 kU_A/L. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc, La Jolla, USA).

Detection of nsLTP in durum wheat extract was performed by IgE Immunoblot inhibition assay as described elsewhere [28]. Briefly, durum wheat extract was subjected to SDS-PAGE (100 µg protein/cm) and transferred to nitrocellulose membrane (Protran 0.2 µm, Fisher

Scientific). After blocking the membrane was incubated over night with a pooled serum (Tab. 1: #26 & #27, 1:8) containing 200 µg/ml of inhibitors (rTri tu 14 and nPru p 3 or, as negative control, BSA). Detection of bound IgE was done using a monoclonal mouse anti-human IgE-AP antibody (1:750) (BD Biosciences, Heidelberg, Germany) and the AP substrate reagent kit (Bio-Rad, Munich, Germany).

Cross-reactivity between the different nsLTPs was evaluated by IgE inhibition ELISA assays as described [28].

Basophil histamine release test

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of a non-allergic donor and IgE was stripped from $Fc_{\varepsilon}RI$ by lactic acid treatment as described elsewhere [29]. Subsequently the cells were passively re-sensitized with patient's serum. Histamine release was performed with both *T. aestivum* and *T. turgidum* extract (1 ng/ml-10 µg/ml) and with purified rTri tu 14, rTri a 14 and nPru p 3 (100 pg/ml-1 µg/ml). Allergen-induced histamine release levels ≥10% of total histamine content of the cells were considered positive.

Results

Recombinant nsLTP from *T. turgidum* shares features with allergenic Tri a 14 and Pru p 3

The nsLTP from *T. turgidum* Tri tu 14 (JF799976) shares 52% amino acid identity (aa-id) with allergenic nsLTP from peach Pru p 3 (AJ277163), as well as 49% and 41% aa-id with two allergenic nsLTP isoforms from *T. aestivum* wheat, Tri a 14.01 (AJ852536) and Tri a 14.02 (FN391139), respectively (**Fig. 1A,B**). Interestingly, NCBI gene bank analysis revealed two nsLTPs from *T. aestivum* leaves (ltp9.2c, CAH69206) and *T. turgidum* seeds (ltp9.2, AJ784903) [30], both encoding proteins showing 100% aa-id with Tri tu 14 (**Fig. 1A**).

The alignment of the 3D-structure models of Tri tu 14, with Tri a 14.01 and Pru p 3 is depicted in **Fig. 1C**. The overlay of 3D-structures indicates that the proportion of conserved amino acids exposed on the surface (red areas) may be higher between Tri tu 14 and Pru p 3 than between Tri tu 14 and Tri a 14 (**Fig. 1C**).

Recombinant Tri tu 14 was generated for diagnostic purpose and allergenicity assessment. A sequential combination of ion exchange and gel filtration chromatography was suitable to prepare a protein with sufficient purity as indicated by a distinct peak of the eluted protein (not shown) and a single band by SDS-PAGE analysis (**Fig. 2A**). The apparent molecular mass of Tri tu 14, as estimated by SDS-PAGE fits with the theoretical mass of around 8.95 kDa, and is comparable with that of rTri a 14.01 and nPru p 3. For rTri a 14.01, the apparent molecular weight is slightly higher than the two others nsLTPs due to terminal tags comprising an additional hexahistidine-tag encoded by the expression plasmid. All three purified nsLTPs showed comparable secondary structure signatures with spectral minima at 208 and 222 nm, which are typical for α-helical structural elements indicating structural integrity and intact 3-dimensional structure (**Fig. 2B**). Accumulation of nsLTPs in wheat extract was verified by MS analysis and IgE competition assays. Notably, both extracts from *T. turgidum* and *T. aestivum* displayed a similar protein pattern with a predominant band at approximately 10 kDa. MS analysis confirmed the protein in *T. turgidum* extract as Tri a 14.01 (Q8GZB0, PLGS protein score 595, 2 tryptic peptides, protein sequence coverage 16,4%, precursor RMS mass error 5,8 ppm), whereas the accumulation of Tri tu 14 could not be verified in this experimental setting. In addition, immunoblot inhibition using Tri tu 14 and Pru p 3 confirmed the presence of an IgE-reactive nsLTP in *T. turgidum* extract (data not shown).

Tri tu 14 displays IgE-reactivity in patients with wheat food allergy and baker´s asthma

In order to explore the frequency of IgE sensitization to Tri tu 14 experimental ImmunoCAP analysis was performed. Remarkably, positive skin reactions to nTri a 14 could not be confirmed by ImmunoCAP testing using Tri a 14 in 7/20 patients with baker´s asthma (**Tab. 1)**. In order to apply uniform inclusion criteria for both patient groups, sera with Tri a 14 positive ImmunoCAP values were selected for further analysis. Specific IgE values for Tri tu 14 were determined by ImmunoCAP testing and were correlated with IgE values for wheat (*T. aestivum*), Tri a 14.01 and Pru p 3.01 (**Tab. 1, Fig. 3; Fig. S1**) in 25 wheat allergic patients: 12 patients with FA/WDEIA, and 13/20 patients with baker's asthma.

Specific IgE values for Tri tu 14 and Tri a 14 revealed overall good correlation considering all patients tested (R=0.929) (**Fig. 3A)**. Interestingly, all patients with FA/WDEIA, but only 77% (10/13) of baker's asthma patients, were sensitized to Tri tu 14 (**Fig. S1B, C**).

Among Tri a 14 sensitized patients 92% (23/25) showed positive serum IgE to wheat, whereas one serum from each patients group was IgE negative to wheat extract (**Fig. 3B, Fig S1A**). Notably, both wheat negative sera were IgE-reactive to Tri tu 14 suggesting an increased diagnostic sensitivity of wheat nsLTPs in comparison to wheat extract. Moreover, 9/12 patients with FA/WDEIA revealed higher specific IgE values for Tri tu 14 (mean=17.14 kU_A/L, median=8.75 kU_A/L) than for wheat (mean=5.40 kU_A/L, median=2.28 kU_A/L) (**Fig. S1C**). These findings were reflected by a weak correlation (R=0.16) between specific IgE levels to wheat extract and Tri tu 14 in patients with FA/WDEIA (**Fig. 3**). In contrast, in patients with baker´s asthma specific IgE values for Tri tu 14 were 7.84 kU_A/L (mean) and 2.75 kU_A/L (median) and wheat 5.14 kU_A/L (mean) and 4.6 kU_A/L (median) (**Fig. 3B, Fig. S1**). Three sera showed IgE-reactivity to wheat but were not reactive with Tri tu 14 indicating other allergens than Tri tu 14 to be involved.

Since the data suggest that sensitization to Tri tu 14 is strongly associated with FA/WDEIA the engagement of Pru p 3 in the sensitization to wheat was further analyzed (**Fig.3 C**). IgE sensitization to Pru p 3 could be detected in 80% (20/25) of all patients, in 69% (9/13) of patients with baker's asthma and in 92% (11/12) of patients with FA/WDEIA (**Fig. S1**).

In the study group (n=25) median IgE-values of 2.9 kU_A/L, 1.8 kU_A/L, 2.6 kU_A/L and 4.4 kU_A/L were calculated for Tri tu 14, Tri a 14, wheat (*T. aestivum*) and Pru p 3, respectively (**Fig. S1**).

In the respective subgroups, in comparison to baker's asthma patients, the group of FA/WDEIA patients revealed higher specific median IgE values for Tri tu 14 (8.8 kU_A/L vs 2.8 kU_A/L), Pru p 3 (5.8 kU_A/L vs 2.1 kU_A/L) and Tri a 14 (3.9 kU_A/L vs 1.6 kU_A/L). By contrast, median wheat specific IgE levels were higher for the subgroup of baker's asthma patients (4.6 kU_A/L) than in the FA/WDEIA subgroup (2.3 kU_A/L) (**Fig. S1B, C**).

Tri a 14-sensitized but asymptomatic individuals (n=7) did not show any significant differences in comparison to wheat allergic patients in terms of the IgE binding properties to wheat, Tri tu 14 and Pru p 3 (**Tab. 1**, data not shown).

Tri tu 14 shows IgE cross-reactivity to other nsLTPs

Initial immunoblot inhibition experiments using a pooled serum revealed substantial IgE cross-reactivity between Tri tu 14 and Pru p 3 (data not shown). Therefore, IgE cross-reactivity between Tri tu 14, Tri a 14 and Pru p 3 was further analyzed using individual sera in dose-dependent ELISA cross-inhibition experiments. Results from two patients, a baker's asthma patient (#15) and wheat food allergic patient (#26), both with strong reactivity to each of the tested nsLTPs (CAP-classes $\geq$4) are depicted in **Fig. 4**.

Using serum #15, and Tri tu 14 on the solid phase, Tri tu 14 itself could outcompete IgE-binding (self-inhibition) up to 100%. When applying Tri a 14 as inhibitor nearly no competition of IgE-binding was observed, whereas Pru p 3 was able to inhibit the binding of IgE to Tri tu 14 up to 70% even at low concentrations (**Fig. 4A**). Limited cross-reactivity between the two wheat nsLTPs was confirmed by using Tri a 14 on the solid phase (**Fig. 4B**). Tri tu 14, but also Pru p 3, did show only up to 20% of inhibitory capacity. In contrast, IgE binding was almost completely abrogated upon pre-incubation of serum with *T. turgidum* and *T. aestivum* extract, indicating the presence of Tri a 14 (cross-) reactive nsTLPs in both wheat seeds. Remarkably, neither any of the wheat nsLTPs nor wheat extracts were shown to outcompete IgE-binding to Pru p 3 (**Fig. 4C**).

Serum #26 displayed deviating cross-reactive properties. Using Tri tu 14 on the solid phase Pru p 3 as well as Tri a 14 and wheat extracts exhibit clear inhibitory capacity indicating the presence of Tri tu 14 (cross-) reactive nsTLP in both wheat seeds (**Fig. 4D**). In contrast to serum #15 all three nsLTPs possess substantial cross-reactive properties indicating overlapping IgE-epitopes for this patient **(Fig. 4E**). Interestingly, IgE cross-reactivity between Pru p 3 and Tri tu 14 (but not between Pru p 3 and Tri a 14) was more pronounced in wheat food allergic patient #26, which was not the case for baker´s asthma patient #15 (**Fig. 4F**). BSA used as negative control showed no unspecific inhibitory activity in all experimental settings.

Basophil histamine release tests indicate allergenic potency of Tri tu 14

To evaluate the allergenic potency of nsLTPs from wheat and peach in wheat allergic patients, *in vitro* mediator release assays were performed using passively sensitized basophils. Tri tu 14 and Pru p 3 were able to trigger a histamine release of up to 20% for

patient #15 (**Fig. 5A**) and up to 60% for patient #26 (**Fig. 5B**). Likewise, Tri a 14 did not show biological potency in patient #15 (**Fig. 5A**) and histamine release up to 20% in patient #26 (**Fig. 5B**). For both, *T. aestivum* and *T. turgidum*, substantial histamine release by extracts was provoked only by using concentrations of >100 ng/ml in patient #26. BSA applied as negative control did not cause any histamine release.

Discussion

Wheat is a potent inhalant and ingestive allergenic source which causes clinical manifestation of baker's asthma, wheat-mediated food allergy and exercise induced anaphylaxis (WDEIA) [6, 16]**.** Although both wheat cultivars, *T. aestivum* (used for bread and bakery products) and *T. turgidum* (used for pasta, pizza, bulgur, semolina and couscous) are known to be involved in allergic reactions [6, 20], allergens have only been identified in *T. aestivum* but not for *T. turgidum.* So far, 27 allergens from *T. aestivum*, including the major allergen nsLTP Tri a 14 [16], are recorded in the database of the WHO/IUIS Allergen Nomenclature Subcommittee. Since (1) nsLTPs are likely conserved in *T. turgidum* and (2) the targeted induction of *T. turgidum* nsLTP expression is considered to increase plant tolerance to abiotic stress and pathogens [19, 31, 32], the allergenicity assessment of nsLTP from *T. turgidum* was the main objective of the present study.

In silico analysis of *T. turgidum* nsLTP Tri tu 14 cDNA revealed two genes, ltp9.2 and ltp9.2c in *T. turgidum* seeds and *T. aestivum* leaves [30], respectively, both encoding proteins with 100% aa-id with Tri tu 14. Nevertheless, the allergenic properties of a protein corresponding to Tri tu 14 have not been characterized so far. Therefore, the cDNA sequence of a nsLTP

from *T. turgidum* (TdLTP4) [19] served as template for the generation of recombinant *T. turgidum* nsLTP. The nsLTP isoform Tri a 14.01 from *T. aestivum* was applied as reference since IgE sensitization to this isoform is described to be more prominent than sensitization to Tri a 14.02 [17], and Tri a 14.01 was already utilized for ImmunoCAP testing.

Thirty-two wheat allergic patients were recruited for the study: 20 Spanish patients with baker's asthma and 12 Italian patients with wheat food allergy or WDEIA. Finally, considering positive Tri a 14-ImmunoCAP values as a consistent inclusion criterion in 25/32 patients, 13 patients with baker's asthma (group 1) and 12 patients with FA/WDEIA (group 2) were enrolled in the study. Wheat allergy was confirmed by bronchial challenge tests with *T. aestivum* in all patients with baker´s asthma (n=13), and by DBPCFC or open food challenge to pasta in all food allergic/WDEIA patients tested (n=9).

By ImmunoCAP analysis nsLTP from *T. turgidum* was identified as an allergen in 88% of the Tri a 14 sensitized patients and was named Tri tu 14 by the WHO/IUIS Allergen Nomenclature Subcommittee. The ImmunoCAP analysis justifies the implementation of Tri tu 14 for component-resolved diagnosis (CRD) in addition to wheat extract to increase the diagnostic sensitivity at least for 2/25 patients. So far, Raulf and co-workers [4] reported whole wheat flour extract rather than single allergens to have superior diagnostic sensitivity in baker´s asthma. The authors hypothesized that, upon inclusion of further allergens, CRD might help to differentiate e.g. between baker´s asthma and wheat-induced food allergy. Of note, since patients of the present study group were pre-selected by sensitization to Tri a 14 we can only speculate about the prevalence of sensitization in patients with baker´s asthma

or food allergy/WDEIA. The data of the present study do not confirm previous reports suggesting that Tri a 14 may to be exclusively associated with wheat-induced respiratory allergy [33].

Our ImmunoCAP results provide some evidence that sensitization to Tri tu 14 is more prominent in wheat FA/WDEIA. All patients with wheat FA/WDEIA showed Tri tu 14 specific IgE (R=0.930), whereas 3/13 patients with baker´s asthma did not (R=0.97). In addition, the data reveal the highest Tri tu 14 specific IgE-level for subjects with wheat FA/WDEIA. The median Tri tu 14 specific IgE value in patients with wheat FA/WDEIA was 8.8 kU_A/L but only 2.8 kU_A/L in baker‘s asthma patients. In addition, sensitization to Pru p 3 was more prominent in wheat FA/WDEIA than in patients with baker´s asthma. Pru p 3 is known to play a pivotal role in food allergy and thought to act as genuine sensitizer.

Further evidence for an engagement of Tri tu 14 in wheat FA/WDEIA was deduced from IgE cross-inhibition experiments. Here, IgE cross-reactivity between Pru p 3 and wheat nsLTPs, in particular Tri tu 14, was clearly demonstrated. In contrast, cross-reactivity between Pru p 3 and Tri a 14 was less prominent in a patient with baker´s asthma. These findings are in line with results of Palacin and co-workers [34] who observed a low IgE cross-reactivity between Tri a 14 and other fruit nsLTPs such as Pru p 3. In line with this, only Tri tu 14 and Pru p 3, but not Tri a 14, were able to induce substantial release of histamine from passively sensitized effector cells in the present study. However, as these data are based on samples from single patients, they have to be interpreted with caution and further studies in larger patient groups are required.

The close association between sensitization to Tri tu 14 and Pru p 3 and the cross-reactivity between the two nsLTPs may be explained by conserved surface patches on these molecules. Although Tri tu 14 shows similar aa-id with Tri a 14 (49%) and Pru p 3 (52%) it seems that Tri tu 14 and Pru p 3 share higher proportion of conserved amino acids on the surface which might define cross-reactive conformational IgE-epitopes. Any conclusion to the importance of Tri tu 14 for patients with wheat FA/WDEIA and its association with Pru p 3 need to be drawn with caution and will require further studies. In our hands confirmatory experiments were limited due to the experimental setting and the volume of serum samples available.

By IgE-immunoblot inhibition using IgE cross-reactive Tri tu 14 and Pru p 3 and ELISA inhibition assays using *T. turgidum* extract the expression of homologous nsLTP in extracts derived from *T. turgidum* seeds could be verified. Subsequently, a nsLTP corresponding to Tri a 14.01 (AJ852536) was identified in *T. turgidum* extract by MS analysis. In this experimental setting we were not able to assess the level of accumulation of Tri tu 14 or isoforms/variants other than Tri a 14.01. Therefore, we can only speculate that the amount of Tri tu 14 might be rather low, and maybe depends on the stage of plant development [30]. Nevertheless, it has to be taken into account, that targeted or stress induced up-regulation of Tri tu 14 or other nsLTPs in durum wheat, implies a risk for allergic reaction for nsLTP-sensitized patients.

In summary, for the first time an allergen from durum wheat (*Triticum turgidum ssp. durum*) has been described as nsLTP Tri tu 14. It was identified as a member of the nsLTP protein family and named nsLTP Tri tu 14. Some evidence suggests that Tri tu 14 rather than Tri a 14 is associated with wheat FA/WEDIA and Pru p 3-mediated food allergy. Moreover, the targeted

expression of Tri tu 14 in wheat crops will increase the allergenicity and risk of unexpected allergic reactions for nsLTP-sensitized individuals.

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Table 1. Demographic and clinical data of selected wheat allergic patients

[1]**As**, asthma; **An**, anaphylaxis; **U**, urticaria; **Gs**, gastrointestinal symptoms; **Ang**, angioedema; **D**, dyspnea; **H**, hypotension; [2]Bronchial challenge test (*T. aestivum*); [3]Open food challenge and [4]DBPCFC (*T. turgidum* pasta); nd, not done.

PATIENTS		CLINICAL SYNDROME	SYMPTOMS[1]	SPT (mm^2)		Provocation	IgE ImmunoCAP (kU_A/L)			
No.	yr/sex			WHEAT	nTri a 14	WHEAT	WHEAT	rTri a 14	rTri tu 14	rPru p 3
01	42/F	BAKER'S ASTHMA	As	20	28	pos[2]	4,86	1,56	0,35	0,38
02	19/M	BAKER'S ASTHMA	As	87	29	pos[2]	5,53	0,36	0,16	0,33
03	19/M	BAKER'S ASTHMA	As, An	22	8	neg[2]	100	0,10	0,10	0,14
04	29/M	BAKER'S ASTHMA	As, An	38,5	32	pos[2]	7,6	4,95	11,80	27,1
05	44/M	BAKER'S ASTHMA	As	18	11	pos[2]	1,6	0,10	0,10	0,10
06	20/F	BAKER'S ASTHMA	As	29	30	pos[2]	4,63	0,95	0,36	0,17
07	32/F	BAKER'S ASTHMA	As	36	26	pos[2]	7,3	1,9	0,10	0,10
08	35/F	BAKER'S ASTHMA	As	28,5	36	pos[2]	4,6	7,18	9,24	22,0
09	19/M	BAKER'S ASTHMA	As	22	22	pos[2]	1,4	6,32	8,08	20,0
10	62/M	BAKER'S ASTHMA	As, U	20	24	pos[2]	2,50	0,37	0,10	0,10
11	16/M	BAKER'S ASTHMA	As, An	38	32	neg[2]	4,2	0,10	0,10	0,10
12	56/M	BAKER'S ASTHMA	As, An	42	46	neg[2]	36,0	0,26	0,27	0,27
13	71/F	BAKER'S ASTHMA	As, An	18	6	neg[2]	3,66	0,10	0,10	0,10
14	26/M	BAKER'S ASTHMA	As, U	28	10	pos[2]	17,2	0,12	0,35	0,53
15	22/M	BAKER'S ASTHMA	As, An	38	42	pos[2]	22,1	25,2	53,90	>100
16	36/M	BAKER'S ASTHMA	As, U	25	26	pos[2]	2,42	1,7	2,85	4,36
17	20/F	BAKER'S ASTHMA	As	26	22	pos[2]	1,56	0,53	0,91	2,1
18	38/M	BAKER'S ASTHMA	As, U	18	12	neg[2]	1,59	0,10	0,10	0,10
19	26/F	BAKER'S ASTHMA	As, U	28	26	pos[2]	2,05	1,25	11,30	8,1
20	25/M	BAKER'S ASTHMA	As	18	18	pos[2]	0,32	0,53	2,75	1,09
21	65/F	Food allergy	Ang, U	nd	nd	pos[4]	1,75	2,74	13,3	2,8
22	46/F	WDEIA	An	nd	nd	pos[3]	2,0	1,79	1,88	1,42
23	26/M	WDEIA	Ang, U	nd	nd	pos[3]	1,42	8,15	12,2	6,94
24	24/M	WDEIA	Ang, U, H	nd	nd	pos[3]	2,82	13,9	19,4	18,8
25	31/M	Food allergy	U, D	nd	nd	pos[4]	1,68	1,37	5,29	4,56
26	37/M	Food allergy	An	nd	nd	nd	2,55	70,7	>100	>100
27	27/F	Food allergy	An	nd	nd	nd	3,61	6,78	34,8	45,1
28	28/M	WDEIA	Ang, U	nd	nd	pos[3]	0,75	1,26	1,31	0,10
29	24/F	Food allergy (BA)	Gs	nd	nd	nd	39,3	0,54	0,93	1,07
30	47/F	Food allergy	U (mild)	nd	nd	pos[4]	0,13	0,75	0,77	3,83
31	65/F	Food allergy	U, Gs	nd	nd	pos[4]	3,49	4,42	14,7	17
32	26/F	Food allergy	U	nd	nd	pos[4]	5,27	15,5	1,07	42,5
33	43/M	NO		nd	nd	nd	1,21	6,97	23,5	37,7
34	40/M	NO		nd	nd	nd	5,48	1,22	2,88	3,81
35	49/M	NO		nd	nd	nd	2,04	0,71	1,24	1,66
36	54/M	NO		nd	nd	nd	2,17	8,25	3,93	13,2
37	28/F	NO		nd	nd	nd	0,14	0,38	0,99	2,03
38	46/M	NO		nd	nd	nd	2,02	1,62	1,26	4,84
39	26/F	NO		nd	nd	nd	2,48	4,56	10,9	15,2

Figure legends

Fig. 1: Amino acid sequence and structural homology analysis of Tri tu 14 from *T. turgidum*. (**A**) Amino acid (aa) sequence identity (%) between Tri tu 14 and allergenic nsLTPs from peach (Pru p 3.0102), two isoforms of Tri a 14 from *T. aestivum*, and ltp9.2/ltp9.2c gene products from *T. turgidum* seed and *T. aestivum* leaves, respectively (GenBank accession numbers are indicated). (**B**) Amino acid sequence alignment of Tri tu 14, Tri a 14.01, and Pru p 3.0102. (**C**) Stereo view of the overlay (PyMOL molecular graphics system) of the 3D-structures of Tri tu 14 (light blue, SWISS-MODEL, maize nsLTP 1AFH as template, 70% aa-identity) with Tri a 14.01 (dark blue, 1GH1) and Pru p 3 (grey, 2ALG). Conserved amino acids exposed on the surface are indicated in red.

Fig. 2: Physicochemical characterization of Tri tu 14. (**A**) SDS-PAGE and Coomassie staining of purified rTri tu 14 (2 µg, lane 1), rTri a 14.01 (2 µg, lane 2), nPru p 3 (2 µg, lane 3), and *T. aestivum* (15 µg, lane 4) and *T. turgidum* extract (15 µg, lane 5). Proteins subjected to MS analysis are indicated. (**B**) CD-spectroscopy of purified nsLTPs. M: molecular weight marker.

Fig. 3: Tri tu 14-specific IgE values in patients with baker´s asthma and wheat food allergy/WDEIA. Comparison of Tri tu 14-specific IgE values with IgE values to Tri a 14 (**A**), wheat (**B**), and Pru p 3 (**C**), as well as between Tri a 14 and Pru p 3 (**D**). Sera from Tri a 14-sensitized and wheat allergic patients (n=25), with manifestation of baker´s asthma (n=13) and wheat FA/WDEIA (n=12) were subjected to ImmunoCAP testing. The overall and subgroup related R values are indicated.

Fig. 4: ELISA IgE cross-inhibition assays. Dose-dependent IgE competition ELISAs were performed with Tri tu 14 (**A & D**), Tri a 14 (**B & E**) and Pru p 3 (**C & F**) on the solid phase. IgE-binding of two sera, one from a patient with baker´s asthma (#15) and one patient with wheat FA/WDEIA (#26) was inhibited by purified nsLTPs (Tri tu 14, Tri a 14, Pru p 3) and extracts from *T. aestivum* and *T. turgidum*, whereas BSA served as negative control.

Fig. 5: Allergenic potency testing of Tri tu 14

Allergenic potency of Tri tu 14, Tri a 14, Pru p 3 and extracts from *T. aestivum* and *T. turgidum* were evaluated by using two sera, one from a patient with baker´s asthma (#15) and one patient with wheat FA/WDEIA (#26) for passive sensitization of human effector cells. BSA served as negative control.

Fig. S1: Wheat-, Tri a 14-, Tri tu 14-, and Pru p 3-specific IgE values of 25 Tri a 14-sensitized wheat allergic patients (A), 13 baker's asthma patients (B) and 12 patients with wheat FA/WDEIA (C). Median IgE levels as well as percentiles of CAP positive (>0.35 kU_A/L) sera are indicated.

Figure 1

A

	Pru p 3 AJ277163	Tri a 14.01 AJ852536	Tri a 14.02 FN391139	ltp9.2 AJ784903	ltp9.2c CAH69206
Tri tu 14 JF799976	52	49	41	100	100
Pru p 3 AJ277163		45	47	52	52
Tri a 14.01 AJ852536			44	49	49
Tri a 14.02 FN391139				41	41
ltp9.2 AJ784903					100

B

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Tri tu 14    avscgqvssalspcisyargngaspsaaccsgvrslassarstadk   43
Tri a 14.01 -idcghvdslvrpclsyvqgg-pgpsgqccdgvknlhnqarsqsdr   44
Pru p 3     -itcgqvssslapcipyvrgggavpp-accngirnvnnlarttpdr   44
             : **:*.* : **: *.:*.   *   **.*::.: . **:  *:

Tri tu 14    qaackciksaa---aglnagkaagiptkcgvsvpyaisssvdcskir  90
Tri a 14.01 qsacnclkgiargihnlnednarsippkcgvnlpytislnidcsr--  89
Pru p 3     qaacnclkqlsasvpgvnpnnaaalpgkcgvsipykisastncatvk  91
            *:**:*:*  :    .:* .:* .:* ****.:** ** . .:*:
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C

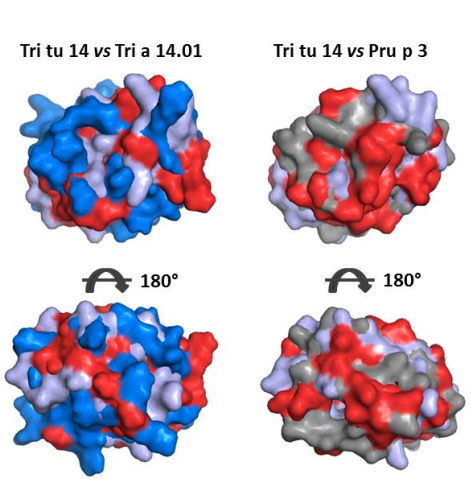

Figure 2

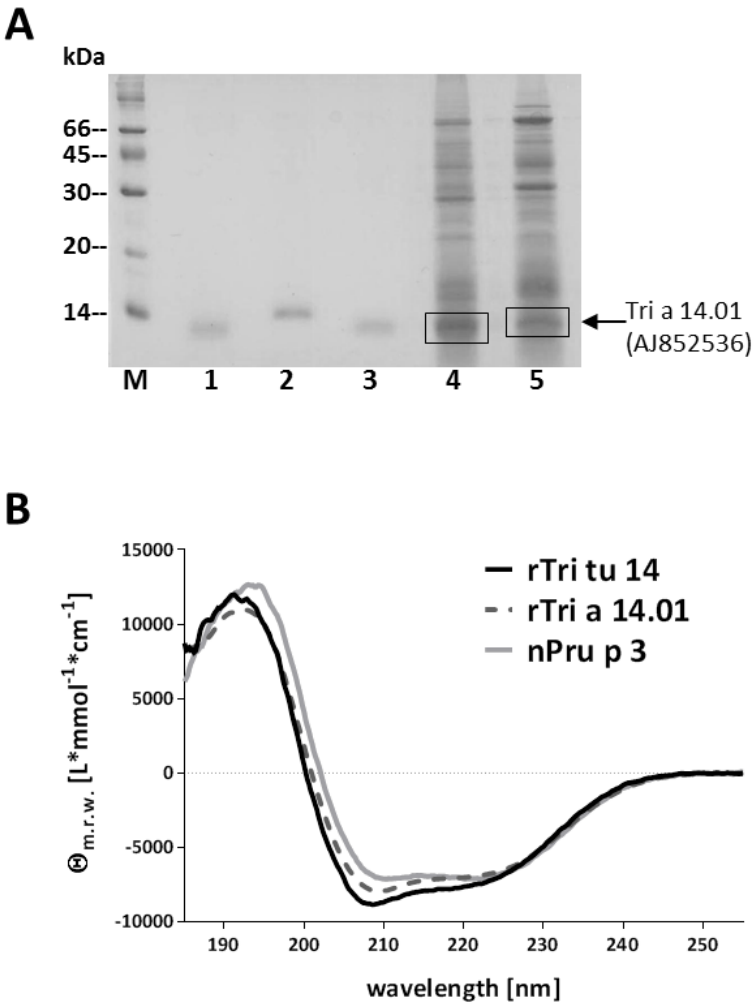

Figure 3

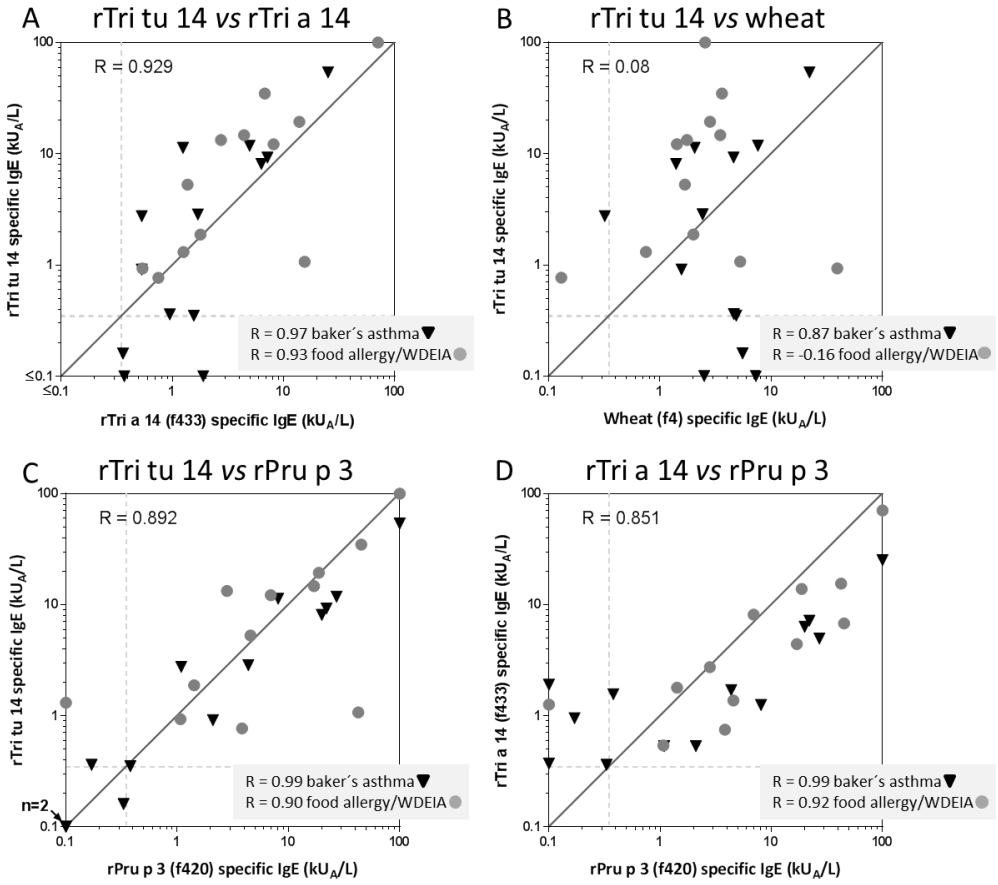

Figure 4

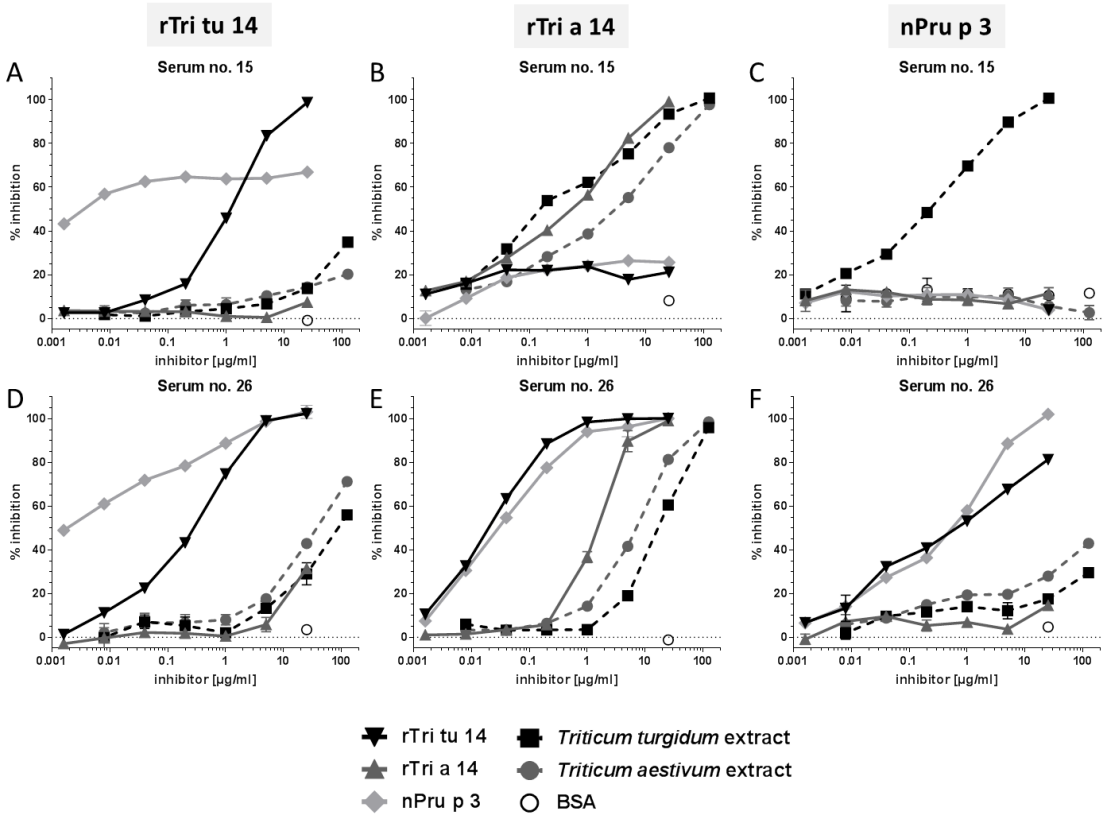

Figure 5