Variability of polymorphic families of three types of xylanase inhibitors in the wheat grain proteome

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Abbreviations used: AC, affinity chromatography; CEC, cation exchange chromatography; CNBr, cyanogen bromide; GH, glycoside hydrolase; λPPase, lambda protein phosphatase; PAA, polyacrylamide; PAbs, polyclonal antibodies; TAXI, Triticum aestivum xylanase inhibitor; TFMS, trifluoromethanesulfonic acid; TLXI, thaumatin-like xylanase inhibitor; XI, xylanase inhibitor; XIP, xylanase inhibiting protein

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ABSTRACT

Cereals contain proteinaceous inhibitors of endo-β-1,4-xylanases (E.C.3.2.1.8, xylanases). Since these xylanase inhibitors (XIs) are only active against xylanases of microbial origin and do not interact with plant endogenous xylanases, they are believed to act as a defensive barrier against phytopathogenic attack. So far, three types of XIs have been identified, i.e. *Triticum aestivum* XI (TAXI), xylanase inhibiting protein (XIP), and thaumatin-like XI (TLXI) proteins. In this study the variation in XI forms present in wheat grain was elucidated using high-resolution 2-DE in combination with LC-ESI-MS/MS and biochemical techniques. Reproducible 2-DE fingerprints of TAXI-, XIP-, and TLXI-type XIs, selectively purified from whole meal of three European wheat cultivars using cation exchange chromatography (CEC) followed by affinity chromatography (AC), were obtained using a pH-gradient of 6 to 11 and a molecular mass range of 10 to 60 kDa. Large polymorphic XI families, not known to date, which exhibit different pI- and/or molecular mass values, were visualised by colloidal CBB staining. Identification of distinct genetic variants by MS/MS-analysis provides a partial explanation for the observed XI heterogeneity. Besides genetic diversity, PTMs, such as glycosylation, account for the additional complexity of the 2-DE patterns.
1 INTRODUCTION

Endo-β-1,4-xylanases (E.C.3.2.1.8, further referred to as xylanases) are crucial enzymes in the breakdown of arabinoxylan, the predominant cell wall non-starch polysaccharide of cereals like wheat [1]. Most of the xylanases are confined to glycoside hydrolase (GH) families 10 and 11 [2].

Little is known about plant endogenous xylanases, which are believed to play a role in cell wall metabolism, seed germination and pollination [3]. In contrast, a large number of microbial xylanases has been described. Micro-organisms synthesize these xylanases, next to other cell wall-degrading enzymes, to provide assimilable nutrients for development. Moreover, xylanases from phytopathogenic species are important virulence factors as they facilitate disintegration of plant cell walls at the host penetration site [4-7]. Several microbial xylanases have been adopted by the paper and pulp industry to reduce the need for chemical bleaching [8] and by the cereal-based food and feed industries to improve processing and/or product quality [9-12].

One of the strategies of plants to try to impede invasion by microbial pathogens is by producing antimicrobial agents [13] such as specific enzyme-inhibiting proteins. These can counteract the action of microbial cell wall-degrading enzymes and hence limit colonisation, as was demonstrated for polygalacturonase inhibitors present in several dicotyledonous plants [14].

In wheat, three types of xylanase inhibitor proteins (XIs) have been discovered over the last decade, i.e. *Triticum aestivum* XI (TAXI) [15], xylanase inhibitor protein (XIP) [16], and thaumatin-like XI (TLXI) [17] which, in view of their specificity for microbial xylanases, and, in the case of TAXI and XIP, their demonstrated inducibility by pathogens [18, 19], most likely classify as plant defence-related proteins.
TAXI-type XIs are a mixture of high-pI inhibitors, TAXI-I to TAXI-IV, with distinct specificities towards xylanases [18, 20]. They occur simultaneously as a ~40 kDa single polypeptide and as a processed form existing of two disulfide-linked polypeptides of ~30 and ~10 kDa [20]. XIP- and TLXI-type XIs are basic, monomeric proteins with a molecular mass of ~30 and ~18 kDa, respectively [16, 17]. Multiple putative TAXI- [18, 21, 22] and XIP-type [19, 23] as well as one TLXI-type gene(s) [17] have been identified in wheat and some have been confirmed. For the three types of XIs, the existence of various forms as well as differences in their spatio-temporal location, due to distinct regulatory control mechanisms, have been suggested. Igawa and co-workers [18, 19] demonstrated that Taxi-III and -IV transcripts mostly accumulate in roots and older leaves, in contrast to Taxi-I. Furthermore they found that expression of Taxi-III and Taxi-IV, in addition to that of Xip-I, is pathogen-inducible. Based on these observations it is speculated that, in analogy with polygalacturonase inhibitors [24], large families of isoforms have adaptively co-evolved with antagonistically active microbial xylanases to achieve a superior counterattack against pathogens. In contrast, Taxi-I transcripts are not induced by infection, suggesting a distinct physiological role in planta [18]. Together, the three types of XIs make up a significant proportion (approx. 2.5%) of the physiologically active albumin/globulin population, present in wheat grain. Thus, it is logical to assume that this group of proteins can be of great meaning for the wheat plant. Their importance in reducing the activity of added microbial xylanases in wheat-based food processes has already convincingly been demonstrated [25-28] and led to the development of inhibitor-insensitive xylanases, less prone to year-to-year wheat inhibitor content variations [29-31].
Despite extensive characterization of TAXI-, XIP-, and TLXI-type XIs, there is a lack of knowledge on their polymorphism in wheat grain. The aim of this study was to elucidate this unknown heterogeneity using high-resolution 2-DE and subsequent MS analysis. For the first time a study was undertaken concurrently for the three types of wheat XIs.
2 MATERIALS AND METHODS

2.1 Materials

Wheat cultivars Claire (harvest 2005), Zohra and Koch (harvest 2003) were obtained from AVEVE (Landen, Belgium) and ground into whole meal using a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden). Grindamyl H640 bakery enzyme, containing a Bacillus subtilis GH family 11 xylanase, was purchased from Danisco (Braband, Denmark). Penicillium purpurogenum GH family 10 xylanase was kindly made available by Prof. Jaime Eyzaguirre (Laboratorio de Bioquimica, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Chile). A GH family 11 xylanase from Aspergillus niger and Xylzyme AX tablets, which comprise azurine cross-linked wheat arabinoxylan, were from Megazyme (Bray, Ireland). All other reagents, BSA, casein, synthetic peptides and bacteriophage \( \lambda \) protein phosphatase (\( \lambda \)PPase) were purchased from Sigma-Aldrich (Bornem, Belgium).

2.2 Extraction of wheat soluble seed proteins

Wheat whole meal was ground in liquid nitrogen using mortar and pestle, and 250 mg fine powder was suspended in 1.0 ml ice-cold extraction buffer [50 mM Tris-HCl pH 7.8, Complete Protease Inhibitor Cocktail (1 tablet/10 ml buffer, Roche Diagnostics, Vilvoorde, Belgium)], incubated for 10 min on ice with intermittent mixing and centrifuged (14000g; 15 min, 4°C). Proteins were precipitated (overnight, -20°C) by addition of 4 volumes 10% TCA in acetone. Pellets were washed twice with 80% acetone and air-dried.

2.3 Purification of xylanase inhibitors from wheat whole meal
Purification of the three types of XIs in wheat whole meal was performed using cation exchange chromatography (CEC) followed by affinity chromatography (AC) with immobilised xylanases according to a protocol described by Gebruers et al. [32] with a few modifications. The procedure was down-scaled and extended storage times, during which the protein population may undergo unwanted modifications, e.g. due to wheat endogenous enzymes, were avoided. TAXI-type proteins were bound to the first AC column, coupled with a GH family 11 B. subtilis xylanase. Isolation of XIP- and TLXI-type proteins was performed in a second affinity-based step, this time with an immobilized GH family 11 A. niger xylanase as biospecific ligand. Protein concentrations were estimated according to Bradford [33] with BSA as standard.

A second, modified procedure for purification of XIs from wheat whole meal was performed to affirm or disaffirm the generation of artefacts during extraction and isolation. Complete Protease Inhibitor Cocktail (1 tablet/ 50 ml buffer) and pepstatin A (35 µg/ 50 ml buffer) were added to the aqueous extraction solution as well as to the eluates of the CEC column. Furthermore, all extraction and purification steps were performed at 7°C and in the shortest time period possible (~3 days).

2.4 2-DE and staining

TCA-acetone pellets of crude wheat soluble proteins (see above) were dissolved in 150 µl lysis buffer (7.0 M urea, 2.0 M thiourea, 4.0% CHAPS, 20 mM DTT, 0.5% IPG pH 6-11 buffer, trace of bromophenol blue) and the protein concentration was measured using the 2D-Quant-kit (GE Healthcare, Uppsala, Sweden) as described by the manufacturer. Affinity-purified XI fractions (see above) were desalted and concentrated to ca. 2.0 mg/ml by means of ultrafiltration using Vivaspin 15R concentrators with a molecular mass cut-off of 5,000 Da (Sartorius AG, Goettingen,
Germany). Forty microgram protein aliquots were fully denatured by addition of lysis buffer.

Immobiline Drystips pH 6-11 (18×0.3×0.5 cm) were reswollen overnight in 340 µl Destreak rehydration solution (GE Healthcare) containing 0.5% IPG buffer. Samples were cup-loaded near the anode and focused at 20°C using the Ettan IPGphor II IEF unit (GE Healthcare). The running parameters for IEF were 500 V (120 min), 500-1000 V (60 min), 1000-10000 V (180 min), and 10000 V (55 min), reaching a total of at least 27 kVh. Prior to SDS-PAGE, the IPG-strips were reduced for 15 min at room temperature (RT) using an equilibration buffer (6.0 M urea, 50 mM Tris-HCl pH 8.8, 2% SDS, 30% glycerol, trace of bromophenol blue) containing 65 mM DTT, followed by an alkylation step of 15 min at RT with the same buffer containing 135 mM iodoacetamide. The IPG strips were then transferred to 15% homogenous polyacrylamide (PAA) gels (25×20×0.1 cm) and SDS-PAGE was performed at 20°C using the Ettan DaltSix vertical electrophoresis system in conjunction with the Tris-glycine buffer system [34]. Protein entry was accomplished at 2 W/gel for 45 min, followed by separation at 17 W/gel for 4.5 h. 2-DE gels were stained with the sensitive CBB G-250 method as described by Candiano et al. [35] or using silver staining based on Blum et al. [36] and scanned via the ImageScanner II system with accompanying Labscan 5.00 software (GE Healthcare).

To selectively visualise the glycoproteins present in 2-DE gels a sequential fluorescence-based staining procedure, comprising the Pro-Q® Emerald 300 Glycoprotein stain and the Sypro Ruby total protein stain (Invitrogen, Carlsbad, CA, USA) was applied according to the manufacturer’s instructions.

2.5 Protein identification by tandem mass spectrometric analysis
Protein spots were picked manually from CBB stained gels, and trypsin-digested according to the method of Shevchenko et al. [37]. Tryptic digests were analyzed by LC-ESI-MS/MS on a LCQ Classic (Thermo Electron, San Jose, CA, USA) ion trap MS equipped with a nano-LC column switching system as described by Dumont et al. [38]. MS/MS data were searched against the Viridiplantae division of the GenBank non-redundant protein database using the Mascot (Matrix Sciences, London, U.K.) and against a custom database using the Sequest (Thermo Electron) algorithm. The latter contained all GenBank plant XI sequences as of 10 October 2007, as well as clustered XI-encoding EST sequences. In addition, recently submitted putative TAXI sequences were added to the custom database. The SEQUEST/MASCOT mass tolerance for parent and fragment ions were +3 and +1 Da, respectively. Carbamidomethylation of Cys and oxidation of Met, Trp and His were set as fixed and variable modifications, respectively. Maximally one missed cleavage was allowed, and the neutral loss of water and ammonia from b- and y-ions was taken into consideration. To allow detection of eventually truncated N- and C-termini, the custom database was subsequently N- and C-terminally ‘ragged’ using DBToolkit version 3.1 [39]. For every ‘parent’ sequence the ‘ragging’ process created a series of subsequences. From each n-th subsequence (with $1 \leq n \leq 30$), the first $n-1$ residues were removed from the N- and C-termini.

2.6 C-terminal and de novo sequence analysis

Cyanogen bromide (CNBr)-fragments were generated for C-terminal analysis [40]. De novo sequence analysis of chemically derivatized peptides was carried out essentially as described previously [41]. Mass analysis was performed on an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF optics [42]. Samples were
prepared by spotting 1 µl of a mixture of sample and matrix (7 mg/ml CHCA in 50%
ACN containing 0.1% TFA) on a stainless steel (192-well) MALDI target plate and
allowed to air-dry at RT. Prior to MALDI-MS analysis, the instrument was externally
calibrated with a mixture of Angiotensin I, Glu-fibrino-peptide B, ACTH (1-17), and
ACTH (18-39). For MS/MS experiments, the instrument was externally calibrated
with fragments of Glu-fibrino-peptide.

2.7 Immunoblot analysis
Polyclonal antibodies (PAbs), specifically interacting with TAXI-, XIP- or TLXI-type
XIs, were obtained by rabbit immunisation as described by Beaugrand and co-workers
[43]. Further purification of the PAbs by AC with immobilised native TAXI-, XIP- or
rTAXI-type inhibitors improved specificity. 2-DE separated proteins were
electroblotted (16V, 40 min) onto an activated Protran (0.45 µm pore size)
nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) and probed with
anti-TAXI, anti-XIP and anti-TLXI PAbs as described before [43].

2.8 Determination of apparent xylanase inhibitor activity
Apparent XI activities of wheat whole meal fractions or run-through fractions of CEC
and AC columns were determined colorimetrically with the Xylazyme AX method as
described by Gebruers et al. [20]. Conversion of XI activities into inhibitor levels was
described by Dornez et al. [44]. The levels of TAXI- and XIP-type inhibitors were
measured using a specific GH family 11 \textit{B. subtilis} xylanase and a GH family 10 \textit{P. purpurogenum} xylanase, respectively.

2.9 Chemical deglycosylation of xylanase inhibitors
Affinity-purified and desalted XIs were lyophilized in small glass vials to create a moisture-free atmosphere for deglycosylation with trifluoromethanesulfonic acid (TFMS) [17, 45]. Briefly, dry sample aliquots (500 µg) were incubated (180 min) on ice with a pre-cooled 10.0% anisole in TFMS solution and neutralized by gradually adding droplets of a 60% pyridine solution, thereby keeping the samples at -15°C in a MeOH/dry ice bath. Prior to 2-DE, pellets were dissolved in lysis buffer (see above).

### 2.10 Enzymatic dephosphorylation of xylanase inhibitors

Affinity-purified and desalted XIs were dephosphorylated using broad spectrum λ-PPase. Forty microgram protein aliquots were prepared in 50 µl λ-PPase buffer (50 mM Tris-HCl pH 7.8, 5.0 mM DTT) and incubated for 24 h at 30°C with 0 (negative control sample) and 800 units of enzyme in the presence of 2.0 mM MnCl₂. Ovalbumin (GE Healthcare) and casein were treated in a similar way and used as positive control samples, while BSA was used as a negative control. After dephosphorylation, proteins were desalted and concentrated by means of ultrafiltration using Microcon YM-3 centrifugal filter units with molecular mass cut-off of 3,000 Da (Millipore, Billerica, MA, USA). Prior to SDS-PAGE and 2-DE analysis, proteins were dissolved in sample buffer (see below) and lysis buffer (see above), respectively.

### 2.11 1-D gel electrophoresis and staining

SDS-PAGE was performed on commercial 20% PAA gels using the PhastSystem unit (GE Healthcare). Proteins were denatured in sample buffer (10% glycerol, 62.5 mM Tris-HCl pH 6.8, 2% SDS (w/v), 5% 2-mercaptoethanol (v/v), trace of bromophenol blue). For serial detection of phosphoprotein and total protein profiles, Pro-Q® Diamond Phosphoprotein gel staining (Invitrogen) and subsequent silver staining were
performed according to the manufacturer’s instructions. Phosphoproteins were visualised with a Typhoon 9400 laser fluorescence scanner (GE Healthcare) at an excitation wavelength of 532 nm and using a 560 nm long pass emission filter.
3 RESULTS AND DISCUSSION

3.1 2-DE of wheat soluble seed proteins

Since all three types of XIs are high-pI proteins [46], high-resolution separation of wheat seed proteins (cultivar Claire, Fig. 1) was realized in a linear alkaline pH-gradient of 6 to 11. SDS-PAGE was achieved on 15% homogenous PAA gels, covering a molecular mass range between 10 and 60 kDa, ideally suited for the separation of the three classes of XIs.

Evaluation of the 2-DE pattern (Image Master 2D-Platinum software, GE Healthcare) resulted in the detection of over a thousand spots. To reveal the presence and location of the three classes of XIs in this complex pattern of wheat seed proteins, 2D-gels were subjected to immunoblotting with PAbs, specifically reacting with TAXI-, XIP- or TLXI-type XIs. Fig. 1 shows that the extraction/precipitation procedure and subsequent 2-DE analysis preserved the three classes of XIs, as immunostaining was observed for the 40 and 30 kDa polypeptides of TAXI-type proteins, as well as for XIP- and TLXI-type XIs. As expected, the 10 kDa C-terminal parts of the cleaved form of TAXI-type proteins escaped this pH-range, given their more acidic pI-values (pI 5.0-5.3) [20].

The large number of spots, detected with western blotting and probing with XI-specific PAbs, was not anticipated. To reveal the large heterogeneity in XIs and, in addition to allow detection and identification of relatively low-abundant forms, a selective enrichment of the target proteins was performed.

3.2 2-DE of isolated polymorphic wheat xylanase inhibitors

3.2.1 Purification of the three types of wheat xylanase inhibitors
To reduce the large number of non-inhibitor proteins present in wheat grain extracts while retaining all different forms of the three classes of XI-proteins, a selective, chromatographic pre-fractionation step was performed.

TAXI-, XIP- and TLXI-type XIs were isolated from wheat whole meal extracts originating from three European wheat cultivars, selected for their distinct XI activities. TAXI and XIP levels, measured *in vitro* by the Xylazyme AX method, were 110, 90 and 155 ppm, and 375, 300 and 325 ppm, for the Claire, Koch and Zohra cultivars, respectively. Following extraction and concentration by CEC, wheat whole meal extracts were applied on a series of two affinity columns. Only members of the TAXI inhibitor class were retained by the *B. subtilis* xylanase, while the *A. niger* xylanase bound the remaining two types of inhibitors.

### 3.2.2 2-DE fingerprints of purified xylanase inhibitors

For affinity-purified, desalted protein fractions, containing almost solely TAXI-type (Fig. 2A) or XIP-/TLXI-type (Fig. 3A) XIs, reproducible high-resolution spot fingerprints were obtained in the pH-gradient 6-11, and with SDS-PAGE on 15% PAA gels. Thus, TAXI-, XIP- as well as TLXI-type inhibitors exhibit a large variability in molecular mass and/or pI within a single wheat cultivar, as was expected from the western blot experiment. Moreover, despite some small differences between these 2-DE fingerprints (Figs. 2A and 3A) and the immunoblotted 2-DE patterns (Fig. 1), possibly due to differences in inhibitor concentration or presence/absence of other wheat seed proteins, the overall spot patterns were very similar, validating the affinity-based purification.

For the spots identified as TAXI-type proteins (see paragraph 3.3.1), only small differences in molecular mass could be detected, while a large variation in pI-values
was visible (Fig. 2A). Estimated molecular masses were 45-46 kDa for the non-cleaved form and 32-33 kDa for the N-terminal polypeptides of processed TAXI-type proteins. These values are slightly higher than expected from their amino acid sequences. A few faint spots with lower molecular masses (41-44 kDa and 25-27 kDa), were observed as well. They may have arisen from partial break-down of the TAXI protein, albeit without major structural changes to the active site as they still bind to the enzymes on the affinity columns. The non-processed form of TAXI-type proteins corresponded to spots with pI-values between ~7.5 and ~9.5, while the pI-range for the N-terminal polypeptides of the processed form varied between ~8.9 and 9.5. In contrast to TAXI-type inhibitors, the spots, identified as XIP-type proteins (see paragraph 3.3.1), showed much greater variability in molecular mass (Fig. 3A). The 2-DE pattern consisted of vertical rows of spots with molecular masses varying between 29 and 36 kDa, which were positioned at pI-values between ~7.2 and ~9.4. The same was true for the spots identified as TLXI proteins (see paragraph 3.3.1), except that there was only one row of spots at pI ~9.8 and within a molecular mass range of 18-21 kDa. As for TAXI-type inhibitors, a few weak spots of XIP-(iso)forms were visible at lower molecular masses.

Furthermore, the 2-DE patterns obtained for the polymorphic families of XIs, present in cultivar Claire, were very similar for the cultivars Koch (Figs. 2B and 3B) and Zohra (Figs. 2C and 3C). About 95% of all XI forms (matched in Image Master 2D-Platinum software) were found in the three cultivars. Spots 45-47 (Fig. 3A) from cultivar Claire were slightly shifted in cultivars Koch and Zohra, possibly because of variable post-translational modifications or (homoeo)allelic variation and, more exceptionally, the cultivar Zohra did not show spots near 18-21 kDa, which implies
that TLXI-type proteins are not present, or only present in undetectable amounts in this cultivar.

3.3 Identification of affinity-purified proteins

3.3.1 Xylanase inhibitor proteins

Using LC-ESI-MS/MS most of the protein spots (Figs. 2A and 3A) were identified as XIs (Table 1, Supplementary Table 1). Moreover, an attempt was undertaken to distinguish between genetic variants, despite their limited differences in amino acid sequences.

Until recently, gene sequences of six TAXI variants have been published, i.e. Taxi-Ia (AJ438880) [21], Taxi-Ib (AJ697851), Taxi-IIa (AJ697849), Taxi-IIb (AJ697850) [22], Taxi-III (AB178471) and Taxi-IV (AB114628) [18]. In addition, six putative wheat TAXI-sequences, Taxi-725ACCN (EU082811), Taxi-725ACC (EU082810), Taxi-725OS (EU082812), Taxi-602OS (EU082813) Taxi-801OS (EU082814) and Taxi-801NEW (EU082815) were made available too.

In total, 24 spots were unequivocally (from 3 up to 20 non-redundant significant peptide hits per spot) identified as TAXI-type proteins (Table 1, Supplementary Table 2). Among these, 13 spots correspond to full-length TAXI form A (Fig. 2A, spots 1-13), and 11 spots to the 30 kDa fragment of form B (Fig. 2A, spots 14-24). The conserved cleavage site separating the 30 and 10 kDa TAXI polypeptides is indicated in Fig. 4. The high amino acid sequence similarity among the TAXI variants and hence the limited amount of variant specific tryptic peptides (Fig. 4), however, often confounded the search engines Mascot and Sequest, thereby complicating the proper assignment of spectra to a specific TAXI variant. Therefore, TAXI variants were tentatively assigned by manually calculating the maximum number of significantly
scored, variant matching peptide hits in each spot. When not all peptides could be matched to a single variant, a second or third tentative assignment of the spectra to other TAXI variants was performed. In this way, all 847 significantly scored tandem MS spectra could be ascribed to minimally 4 out of 12 TAXI sequence variants, present in our customized database, being TAXI-Ia, TAXI-IIa, TAXI-IV and TAXI-725ACCN. The occurrence of the variant TAXI-IIb (one specific peptide hit) cannot be excluded because only a two amino acid difference exists between TAXI-IIb and TAXI-IV, resulting in just two detectable distinct tryptic peptides. The same is true for TAXI-725ACC and TAXI-725ACC, for which eight differences in amino acids exist, giving rise to only five detectable distinct tryptic peptides. None of the TAXI-725ACC specific peptides was observed, however. They could all be accounted for as originating from TAXI-725ACCN. Tryptic peptides specific for TAXI-Ib or TAXI-III, which show 99.6% identity, were not found in the corresponding 2-DE spot patterns. To date, TAXI-Ib has only been produced recombinantly in Pichia pastoris [22], while Taxi-III transcripts were only demonstrated to occur in lemma, palea and leaves of the wheat plant after pathogen inoculation [18]. It should be noted that the presence of other highly similar TAXI sequence variants cannot be ruled out either. After all, our data show that single spots often contain different TAXI sequence variants, and that each TAXI variant occurs in several different spots (Table 1).

Regarding the identification of XIP-type proteins, the situation was less complex (Table 1). Full-length gene sequences were already described for Xip-I (Q8L5C6) [23] and Xip-III (BAD99103) [19]. Most recently Takahashi-Ando and co-workers [47] revealed the existence of Xip-R1 (BAF74363) and Xip-R2 (BAF74364) genes. In our analyses, the presence of both XIP-I (Fig. 3A, spots 30, 32-51) and XIP-III (Fig. 3A, spots 25-29, 31) in the 2-DE pattern was confirmed, while none of the MS/MS spectra
could be matched to XIP-R1 or XIP-R2. The latter two XIP-type family members probably reside in wheat plant parts, other than the caryopsis, or occur under other (stress) conditions. In total, 27 spots were unequivocally determined as XIP proteins (Supplementary Table 3). For the third class of XIs, all observed spots (Fig. 3A, spots 52-55) correspond to the only TLXI encoding gene sequence (Table 1) thus far identified in wheat [17].

Most of the XI forms migrated to positions in the 2-DE gel which were in agreement with their theoretical pI-values, e.g. TAXI-725ACCN and XIP-III forms, which have the lowest theoretical pI-values among the XI proteins, were situated close to the neutral part of the pH-range.

Most prominent in the identification of different genetic variants was the observation that the number of protein spots in the 2-DE patterns of all three classes of XIs highly exceeded the number of distinguished genetic variants. To check the possibility that the large variation was caused by proteolytic activity or other side reactions during the protein isolation, the extraction/purification of the three classes the XIs was carried out again for the cultivar Claire. This time a mixture of protease inhibitors was added and the temperature was reduced to prevent the formation of artificial products as much as possible. Comparison of the 2-DE fingerprints, acquired for the multiple (iso)forms of the three classes of XIs, didn’t reveal differences between the outcomes of the standard and the modified purification procedure (results not shown), implying that no artefacts were produced either due to endogenous proteolytic activity or enzymatic side reactions. Hence, the large heterogeneity in inhibitor forms is most likely caused in planta by PTMs. It can not be excluded, however, that other XI gene sequences exist, which are thus far unknown because of the size and complexity of the hexaploid, not yet fully sequenced, wheat genome.
XI- and XIP-type XIs seem to be organized in multigene families. These observations fit well with their suggested role as plant defence-related proteins and are in line with observations on polygalacturonase inhibitors, which evolved as large families with specific recognition abilities against the many polygalacturonases produced by phytopathogenic fungi [24]. Thus far, different xylanase specificities of TAXI-I- and TAXI-II-type XIs have been demonstrated [20]. It is thus not unlikely that XIs too underwent a co-evolution with their pathogenic counterparts, resulting in the presence of a large heterogeneity in expressed forms, conferring enhanced resistance to multiple pathogens [48]. Igawa and co-workers [18] provided evidence for induced expression of Taxi-III and Taxi-IV in lemma/palea or leaves upon infection with F. graminearum and E. graminis, while expression of Taxi-I is only up-regulated in response to abiotic stress. Furthermore it has been demonstrated that Xip-I and Xip-R1, but not Xip-III and Xip-R2, are strongly transcribed in infected wheat leaves, though this appears to be pathogen-dependent [19, 47]. Wounding, as well as treatment of leaves with methyl jasmonate, also enhance the expression of Xip-I [19]. Accordingly, it is hypothesized that, within the large polymorphism, some XI forms are basal pre-existing defence-related proteins, while others have a more specialized protective role triggered by specific biotic or abiotic stimuli [19, 47].

3.3.2 Non-xylanase inhibitor proteins

From Fig. 2A it can be deduced that, next to spots corresponding to TAXI-type inhibitors, the XI protein preparation also contained some impurities, co-purified on the B. subtilis affinity matrix (Supplementary Table 1). Among these, a bifunctional
α-amylase inhibitor, a class II chitinase, LMW glutenin subunits, a thaumatin-like protein TLP7, β-glucosidases and some unidentified proteins were coupled, probably by non-specific interactions. In the area near neutral pI (6.0-7.0) and low molecular mass (< 18 kDa), a small group of intense spots could be matched by MS/MS to α-amylase inhibitors (Table 1). Their high abundance in the purified XI fraction was surprising. In contrast to other impurities present, the pattern of the α-amylase inhibitors remained unaltered, irrespective of purification scheme or wheat cultivar (Fig. 2). It is not yet clear whether these proteins interact with the B. subtilis xylanase or with TAXI-type inhibitors, and whether they possess any XI activity in addition to their α-amylase inhibitor activity.

3.4 Post-translational modifications

The high multiplicity of spots, identified as the same gene product but differing in molecular mass and/or pI, supports the occurrence of different PTMs for the three types of XIs, independent of wheat cultivar. In order to gain more insight into the post-translational heterogeneity of the polymorphic families of wheat XIs, some of the most commonly occurring PTMs were examined.

3.4.1 Spots with a different molecular mass and the same pI

XIP- and TLXI-type XIs show vertical rows of spots in their 2-DE patterns, indicative for varying degrees of glycosylation, whereas less variation in molecular mass is seen for TAXI-type proteins. TAXI-type XIs have a predicted N-glycosylation site at Asn\(^{105}\) (TAXI-Ia and TAXI-725ACCN) or Asn\(^{107}\) (TAXI-IV/IIb and TAXI-IIa) (Fig. 4) [48]. XIP-I/XIP-III and TLXI have a Asn-X-Ser/Thr motif at positions 89 and 95, respectively [17, 49].
To reveal the non-glycosylated ‘parent’ 2-DE pattern for the three types of XIs, chemical deglycosylation of the affinity-purified proteins was accomplished using TFMS. As expected for XIP- and TLXI-type proteins (Fig. 5B), the vertical trains of spots disappeared due to the acid treatment. For TAXI-type proteins (Fig. 5A) no differences in molecular mass were seen, however, a noticeable shift in pI was observed upon deglycosylation. This was even more so the case for XIP- and TLXI-type proteins. One reason for this shift towards the cathode upon deglycosylation may be the removal of negatively charged sialic acid residues which are possibly build-in as part of the complex carbohydrate structure [51]. A pathway for sialylation was only recently discovered in plants [50] and, moreover, for TLXI, the incorporation of one sialic acid residue in the glycan structure has been described [17]. Although it can not be taken for granted, it has been demonstrated that the effect of TFMS, in the presence of anisole as scavenger, is sufficiently specific, in the sense that the protein backbone and the PTMs, other than glycosylation are stable during the treatment [45, 52].

To complement the above results, TAXI- and XIP-/TLXI-type proteins were, before and after deglycosylation, stained with the fluorescent Pro-Q Emerald 300 glycoprotein stain. The small signal for TAXI-type proteins (Fig. 6A) disappeared upon deglycosylation, while the intense glycoprotein signal of XIP- and TLXI-type inhibitors (Fig. 6B) remained only slightly visible (result not shown). The residual fluorescence may have been due to the presence of N-acetylhexosamine of N-linked glycans that escapes removal by TFMS [45]. Post-staining with Sypro Ruby confirmed the presence of XI spots in gels giving no Pro-Q Emerald signal.

3.4.2 Spots with a different pI and the same molecular mass
In the case of TAXI- (Fig. 2) and XIP-type (Fig. 3) inhibitors, all genetic variants, identified by MS, emerge as multiple spots with distinct pI-values. Conversely, TLXI-type (Fig. 3) proteins were not modified in a way that alters the pI. It could thus be postulated that at least some of the TAXI- and XIP-type XIs are phosphorylated whereas TLXI-type inhibitors are not.

For this purpose, prior to 2-DE, purified TAXI- and XIP-/TLXI-type XIs were treated with λPPase, which acts on all currently known phosphorylated amino acid residues. The dephosphorylated protein patterns for TAXI-, as well as XIP- and TLXI-type XIs (results not shown), were identical to the ones obtained before. This result was confirmed by comparison of dephosphorylated and intact XIs with phosphorylated (casein and ovalbumin) and non-phosphorylated (BSA) control proteins using 1D-SDS-PAGE and Pro-Q-Diamond phosphostaining (Fig. 7A), followed by silver staining (Fig. 7B). From these experiments, we can conclude that phosphorylation does not contribute to the complexity of the XI spot patterns, in particular to differences in pI. Modifications such as acetylation, methylation, deamidation and sialylation all may give rise to cathodic shifts in 2-DE. Examination of these options will require more extensive biochemical analyses.

3.4.3 Micro-heterogeneity at the C-or N-terminal end of the amino acid chain

Terminal truncation was investigated by including systematically N- or C-terminally shortened TAXI and XIP sequence variants in the Sequest database. This way, deletions of 1 or 2 amino acids at the TAXI N-terminus were frequently observed by ESI-MS/MS (Supplementary Table 2). In contrast, C-terminal peptides, if observed, were untruncated.
To further verify whether the different XI-forms are processed *in planta*, CNBr-fragments from multiple spots were generated and analyzed by MALDI-MS and MS/MS analysis [34]. As an example, MS analysis of the CNBr-fractions in spot 2 (Fig. 2A) reveals three peptides with respective m/z values of 1574.85, 1849.08 and 2160.22 Da. The first two correspond to internal CNBr-fragments of the TAXI-725ACCN isoform (both with a homoserinelactone derivative, Δm = -48 Da) while the mass of the fragment at m/z 2160.22 is in full agreement with the theoretical mass of the C-terminal fragment Glu364-Leu382 (calculated molecular mass 2159.14 Da).

In spot 3 (Fig. 2A), three CNBr-fragments at m/z values of 1791.05, 2254.37 and 2160.21 Da were also observed. The two former represent internal fragments indicative for TAXI-Ia (Fig. 4), while the latter coincides with the intact C-terminus. The C-termini of some TAXI proteins were also identified by *de novo* sequence analysis of chemically derivatized tryptic peptides. As an example, in spot 1 and 2, both identified as TAXI-725ACCN, the C-terminal tryptic peptide, LGFSRLPHFTCGGL (Leu368-Leu382) (Fig. 4) was seen by MS/MS analysis. CNBr-fragments were also derived for a number of the XIP-type proteins before and after deglycosylation, since MS often fails to detect glycosylated peptides. In spots 34 and 44 (Fig. 3A), two main signals at m/z 2793.3 and 3037.4 Da were observed after CNBr-cleavage. These two fragments correspond to the C- and N-terminal fragments of the XIP-I-isoform, as deduced from MS/MS analysis and partial N-terminal sequence analysis, respectively (results not shown). After deglycosylation, the same m/z values of the XIP-I-isoform were observed in spots 2-4 (Fig. 5B), with an additional fragment at 1264.6 Da. MS/MS analysis of the latter indicated that it contains the N-terminal sequence, probably generated by a non-specific cleavage. This N-terminal fragment was also present in spot 1 (Fig. 5B) with a satellite peak at
$m/z$ 1344.5 ($\Delta M$ 80 Da). Together, this illustrates that neither C- nor N-terminal processing is responsible for the different horizontal position of the XI spots which share an identical MS identification.
CONCLUDING REMARKS

In the present study, 2-DE and subsequent tandem MS analysis were successfully used to reveal the presence of complex polymorphic XI families in wheat grain and, in addition, to gain insight in the genetic variability present. Moreover, this is the first paper with a simultaneous emphasis on the three XI classes, currently found in wheat. Thanks to a refined pre-fractionation step and the availability of improved basic pH-gradient protocols, it was possible to effectively focus on a small, but from a plant physiological and a wheat processing point of view, very interesting part of the wheat grain proteome.

Although multiple XI genes were already available in public databases, we were able to show that not all of them are actually expressed in wheat grains, or at least not to the same extent. For instance, no variant specific tryptic peptides of TAXI-III/Ib or XIP-R1/R2 were found, while the putative TAXI-725ACCN and XIP-III variants, for which it was not yet known whether expression actually occurred in the mature wheat caryopsis, could be identified in several (major) spots. This underlines the need for integration of data on genomic and proteomic levels. The proteomic approach also enabled us to investigate some ubiquitous PTMs. Glycosylation is responsible for the variation in molecular mass, observed for XIP- and TLXI-type proteins. Some genetic variants of TAXI- and XIP-type proteins display differences in pI, which could not be attributed to phosphorylation or processed C- or N-termini. The existence of yet unknown wheat XI gene sequences can not be excluded.

When looking down the road, the obtained results, including the 2-DE fingerprints of TAXI-, XIP-, and TLXI-type proteins, will be instrumental in exploring the temporal and spatial distribution of XIs in wheat grains by analyzing successive developmental/germination stages and different milling fractions or kernel tissues, respectively.
Additionally, 2-DE analysis of wheat, infected with ubiquitous cereal pathogens, e.g. *F. graminearum*, can provide useful information on the physiological role of TAXI-, XIP-, and TLXI-type (iso)forms in plant defence. More research on such proteins, important for plant resistance, can pave the way for the development of efficient strategies in environment-sound plant protection.

In conclusion, next to providing insight in the variability of polymorphic XI families, this work contributes to a better understanding of the link between XI proteins and XI genes, effectively expressed in wheat. It further provides a strong basis for the analysis of the temporal and spatial distribution of XIs in wheat and of the presumed physiological role of TAXI-, XIP-, and TLXI-type (iso)forms in plant defence.
ACKNOWLEDGEMENTS

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REFERENCES


New TAXI-type xylanase inhibitor genes are inducible by pathogens and wounding in hexaploid wheat, *Plant Cell Physiol.* 2004, 45, 1347-1360.


FIGURE CAPTIONS

Figure 1. Silver stained 2-DE pattern (pH 6-11; 15% PAA gel) of low salt extractable wheat seed proteins (cultivar Claire, 350 µg). Rectangles represent particular regions containing the three types of XIs, visualised by western blotting and probing of membranes with specific anti-TAXI, anti-XIP or anti-TLXI PAbs.

Figure 2. Colloidal CBB stained 2-DE patterns (pH 6-11; 15% PAA gels) of TAXI-type XIs (40 µg) purified from the wheat cultivars Claire (A), Koch (B) and Zohra (C). Numbered spots (A) were excised and analysed by LC-ESI-MS/MS (Table 1 and Supplementary Table 1).

Figure 3. Colloidal CBB stained 2-DE patterns (pH 6-11; 15% PAA gels) of XIP- and TLXI-type XIs (40 µg) purified from the wheat cultivars Claire (A), Koch (B) and Zohra (C). Numbered spots (A) were excised and analysed by LC-ESI-MS/MS (Table 1 and Supplementary Table 1).

Figure 4. Amino acid sequence alignment of TAXI-type XIs, without signal sequence (Clustal W, EBI, default parameters). (*) indicates the conserved cleavage site separating the 30 and 10 kDa TAXI polypeptides. Underlined amino acids are LC-ESI-MS/MS sequenced peptide fragments (in agreement with Supplementary Table 2). CNBr-fragments are indicated in italics.

Figure 5. Colloidal CBB stained 2-DE patterns (pH 6-11; 15% PAA gels) of chemically deglycosylated TAXI-type (A) and XIP-/TLXI-type XIs (B) (cultivar Claire, 40 µg). Arrows indicate the shift in pI after deglycosylation, compared to the
original 2-DE patterns (Figs. 2A and 3A). Numbered spots (1-4) were submitted to C-terminal analysis.

**Figure 6.** 2-DE patterns (pH 6-11; 15% PAA gels) after staining with the fluorescent Pro-Q Emerald 300 glycoprotein gel stain, showing glycosylated proteins in TAXI-type (A) and XIP-type XIs (B) of wheat cultivar Claire (40 µg).

**Figure 7.** SDS-PAGE profile (under reducing conditions) of XI proteins, stained with Pro-Q Diamond phosphoprotein gel stain (A) and subsequently, using the sensitive silver staining (B) procedure. Casein and ovalbumin were used as positive control samples, while BSA was applied as a negative control. TAXI-type XIs (0.4 µg, 1); XIP/TLXI-type XIs (1.0 µg, 2); TLXI-type XIs (0.4 µg, 3); casein (0.4 µg, 4); ovalbumin (0.4 µg, 5); BSA (0.4 µg, 6).
### Table 1. Tandem MS identification of spots, excised from 2-DE gels (pH 6-11; 15% PAA gels) of affinity-purified proteins originating from whole meal of wheat cultivar Claire.

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<th>Protein Name</th>
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<sup>a)</sup> Spot ID as indicated in Figs. 2A and 3A.

<sup>b)</sup> TAXI variants, tentatively assigned by manually calculating the maximal number of significantly scored, variant matching peptide hits in each spot (see Supplementary Table 2).

<sup>c)</sup> XIP variants, tentatively assigned by manually calculating the maximal number of significantly scored, variant matching peptide hits in each spot (see Supplementary Table 3).

<sup>d)</sup> Significantly scored peptides and Sequest/Mascot hits listed in Supplementary Table 1. Sequest cross correlation score $\geq 3.5$ for triply charged peptide ions; Sequest cross correlation score $\geq 2.5$ for doubly charged peptide ions; Sequest cross correlation score $\geq 1.8$ for singly charged peptide ions; Mascot Expect value $\leq 0.05$. 

---

TAXI-type xylanase inhibitors (40 kDa polypeptides)

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XIP-type xylanase inhibitors

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TLXI-type xylanase inhibitors

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α-amylase inhibitors

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**Notes:**

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---

37
Figure 1.

6 11

pI

60 kDa

10 kDa

TAXI (40 kDa)

XIP

TLXI

TAXI (30 kDa)
Figure 2.
**Figure 4.**

TAXI-IIb

EGLPVLAPVTGTKDTATSLYIFPFDHGANLVLDVGPMILWSTCDGGQPPAEIPCSSPTCLLA 60

TAXI-IV

KGLPVLAPVTGTKDTATSLYIFPFDHGANLVLDVGPMILWSTCDGGQPPAEIPCSSPTCLLA 59

TAXI-IIb/III

KGLPVLAPVTGTKDTATSLYIFPFDHGANLVLDVGPMILWSTCDGGQPPAEIPCSSPTCLLA 60

TAXI-IIa

KGLPVLAPVTGTKDTATSLYIFPFDHGANLVLDVGPMILWSTCDGGQPPAEIPCSSPTCLLA 60

TAXI-725ACCN

--LPVLAPVTGTKDTATSLYIFPFDHGANLVLDVGPMILWSTCDGGQPPAEIPCSSPTCLLA 58

TAXI-725ACC

KGLPVLAPVTGTKDTATSLYIFPFDHGANLVLDVGPMILWSTCDGGQPPAEIPCSSPTCLLA 60

TAXI-Ia

--LPVLAPVTGTKDTATSLYIFPFDHGANLVLDVGPMILWSTCDGGQPPAEIPCSSPTCLLA 58

**********.************.******** ******:*:*.*.*****:*.*:********:**

TAXI-IIb

NAYPAPCPAPCGSRDHKFCAYYPYPVTCAGACAGSLHFTFVANTTDGKPVSKVNV 120

TAXI-IV

NAYPAPCPAPCGSRDHKFCAYYPYPVTCAGACAGSLHFTFVANTTDGKPVSKVNV 118

TAXI-Ib/III

NAYPAPCPAPCGSRDHKFCAYYPYPVTCAGACAGSLHFTFVANTTDGKPVSKVNV 120

TAXI-IIa

NAYPAPCPAPCGSRDHKFCAYYPYPVTCAGACAGSLHFTFVANTTDGKPVSKVNV 120

TAXI-725ACCN

NAYPAPCPAPCGSRDHKFCAYYPYPVTCAGACAGSLHFTFVANTTDGKPVSKVNV 118

TAXI-725ACC

NAYPAPCPAPCGSRDHKFCAYYPYPVTCAGACAGSLHFTFVANTTDGKPVSKVNV 120

TAXI-Ia

NAYPAPCPAPCGSRDHKFCAYYPYPVTCAGACAGSLHFTFVANTTDGKPVSKVNV 118

**********.************.*.******** ****:****:****:********:****:**

TAXI-IIb

GVVAACAPSKLLASLPRGSTGVAGLADSGLALPAQVASAQK VANRFLLCLPTGGPVSKVNV 180

TAXI-IV

GVVAACAPSKLLASLPRGSTGVAGLADSGLALPAQVASAQK VANRFLLCLPTGGPVSKVNV 178

TAXI-Ib/III

GVLAACAPSKLLASLPR GSTGVAGLADSGLALPAQVASTQKVANRFLLCLPTGGPVSKVNV 180

TAXI-IIa

RVLAACAPSK LLASLPR GSTGVAGLADSGLALPAQVASAQK VANKFLLCLPTGGPVSKVNV 180

TAXI-725ACCN

GVLAACAPSK LLASLPR GSTGVAGLADSGLALPAQVASKVAKRFLLCLPTGGPVSKVNV 178

TAXI-725ACC

GVLAACAPSK LLASLPR GSTGVAGLADSGLALPAQVASKVAKRFLLCLPTGGPVSKVNV 180

TAXI-Ia

GVLAACAPSK LLASLPR GSTGVAGLADSGLALPAQVASKVAKRFLLCLPTGGPVSKVNV 178

*******:**********:******** ****:*:*.*:******:********:*******

TAXI-IIb

GGGPLPWPQFTSQMDTPLVAKGGPAHYISLKSIKVENTVPVSEERALATGVMWLSTRL 240

TAXI-IV

GGGPLPWPQFTSQMDTPLVAKGGPAHYISLKSIKVENTVPVSEERALATGVMWLSTRL 237

TAXI-IIb/III

GGGPLPWPQFTSQMDTPLVAKGGPAHYISLKSIKVENTVPVSEERALATGVMWLSTRL 240

TAXI-IIa

GGGPLPWPQFTSQMDTPLVAKGGPAHYSARIKVENTVPVSEERALATGVMWLSTRL 240

TAXI-725ACCN

GGGPLPWPQFTSQMDTPLVAKGGPAHYISLKSIKVENTVPVSEERALATGVMWLSTRL 237

TAXI-725ACC

GGGPLPWPQFTSQMDTPLVAKGGPAHYISLKSIKVENTVPVSEERALATGVMWLSTRL 240

TAXI-Ia

GGGPLPWPQFTSQMDTPLVAKGGPAHYSARIKVENTVPVSEERALATGVMWLSTRL 238

*********:******:************:********:********:********:********

TAXI-IIb

PYVLLAPDPYRFPVDAFTKALAAQPAN*GAPVARAVKPVAPFELCYDTKSLGNNLGGYWVP 300

TAXI-IV

PYVLLAPDPYRFPVDAFTKALAAQPAN*GAPVARAVKPVAPFELCYDTKSLGNNLGGYWVP 297

TAXI-IIb/III

PYVLLAPDPYRFPVDAFTKALAAQPAN*GAPVARAVKPVAPFELCYDTKSLGNNLGGYWVP 300

TAXI-IIa

PYVLLAPDPYRFPVDAFTKALAAQPAN*GAPVARAVKPVAPFELCYDTKSLGNNLGGYWVP 300

TAXI-725ACCN

PYVLLAPDPYRFPVDAFTKALAAQPAN*GAPVARAVKPVAPFELCYDTKSLGNNLGGYWVP 298

TAXI-725ACC

PYVLLAPDPYRFPVDAFTKALAAQPAN*GAPVARAVKPVAPFELCYDTKSLGNNLGGYWVP 300

TAXI-Ia

PYVLLAPDPYRPLDAFTKALAAQPAN*GAPVARAVKPVAPFELCYDTKSLGNNLGGYWVP 298

**:**:**:**********:********:********:**:*****:****:****:****
TAXI-IIb  NVGLAVDGSD-WAMTGKNSMDVKPGTACVAFVEMKGVEAGDGRAPAVILGGAQMEDFV  359
TAXI-IV  NVGLAVDGSD-WAMTGKNSMDVKPGTACVAFVEMKGVEAGDGRAPAVILGGAQMEDFV  356
TAXI-Ib/III NVGLAVDGSD-WAMTGKNSMDVKPGTACVAFVEMKGVEAGDGRAPAVILGGAQMEDFV  359
TAXI-IIa  NVLLELDGSD-WALTGKNSMDVKPGTACVAFVEMKGVDAGDGSAPAVILGGAQMEDFV  359
TAXI-725ACCN NVQLALDGSDTWMTGKNSMDVKPGTACVAFVEMKGVEAGDGRAPAVILGGAQMEDFV  358
TAXI-725ACC NVQLGLDGSDTWMTGKNSMDVKPGTACVAFVEMKGVEAGDGRAPAVILGGAQMEDFV  360
TAXI-Ia  NVQLGLDGSD-WMTGKNSMDVKPGTACVAFVEMKGVEAGDGRAPAVILGGAQMEDFV  357

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TAXI-IIb  LDFDMERKRLGFSRFLPQFTGCSSFNFAST  389
TAXI-IV  LDFDMERKRLGFSRFLPQFTGCSSFNFAST  386
TAXI-Ib/III LDFDMERKRLGFLRLPHFTGCGS-------  382
TAXI-IIa  LDFDMERKRLGFLRLPHFTGCGS-------  389
TAXI-725ACCN LDFDMERKRLGFSRFLPHTGCGS-------  382
TAXI-725ACC LDFDMERKRLGFSRFLPHTGCGS-------  384
TAXI-Ia  LDFDMERKRLGFSRFLPHTGCGS-------  381

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Figure 5.
Figure 6.
Figure 7.