Mol Genet Genomics (2015) 290:39–54
DOI 10.1007/s00438-014-0894-5

ORIGINAL PAPER

Biochemical and molecular characterization of *Avena* indolines and their role in kernel texture

Laura Gazza · Federica Taddei · Salvatore Conti · Gloria Gazzelloni · Vera Muccilli · Michela Janni · Renato D'Ovidio · Michela Alfieri · Rita Redaelli · Norberto E. Pogna

Received: 6 March 2014 / Accepted: 31 July 2014 / Published online: 15 August 2014
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Abstract Among cereals, *Avena sativa* is characterized by an extremely soft endosperm texture, which leads to some negative agronomic and technological traits. On the basis of the well-known softening effect of puroindolines in wheat kernel texture, in this study, indolines and their encoding genes are investigated in *Avena* species at different ploidy levels. Three novel 14 kDa proteins, showing a central hydrophobic domain with four tryptophan residues and here named vromindoline (VIN)-1,2 and 3, were identified. Each VIN protein in diploid oat species was found to be synthesized by a single *Vin* gene whereas, in hexaploid *A. sativa*, three *Vin-1,* three *Vin-2* and two *Vin-3* genes coding for VIN-1, VIN-2 and VIN-3, respectively, were described and assigned to the A, C or D genomes based on similarity to their counterparts in diploid species. Expression of oat vromindoline transgenes in the extra-hard durum wheat led to accumulation of vromindolines in the endosperm and caused an approximate 50 % reduction of grain hardness, suggesting a central role for vromindolines in causing the extra-soft texture of oat grain. Further, hexaploid oats showed three orthologous genes coding for avenoindolines A and B, with five or three tryptophan residues, respectively, but very low amounts of avenoindolines were found in mature kernels. The present results identify a novel protein family affecting cereal kernel texture and would further elucidate the phylogenetic evolution of *Avena* genus.

Keywords *Avena* · Kernel texture · Indoline proteins · Wheat transformation

Electronic supplementary material The online version of this article (doi:10.1007/s00438-014-0894-5) contains supplementary material, which is available to authorized users.

L. Gazza (✉) · F. Taddei · S. Conti · G. Gazzelloni · N. E. Pogna
Consiglio per la Ricerca e la Sperimentazione in Agricoltura (CRA-QCE), Via Cassia, 176, 00191 Rome, Italy
e-mail: laura.gazza@entecra.it

V. Muccilli
Department of Chemical Sciences, University of Catania, Via Andrea Doria 6, Catania, Italy

M. Janni · R. D'Ovidio
Department DAFNE, University "La Tuscia", Via C. De Lellis snc, Viterbo, Italy

Present Address:
M. Janni
CNR-Istituto di Genetica Vegetale, Via Amendola 165/A, Bari, Italy

M. Alfieri · R. Redaelli
Consiglio per la Ricerca e la Sperimentazione in Agricoltura (CRA-MAC), Via Stezzano 24, Bergamo, Italy

Introduction

The genus *Avena* likely originated in Asia Minor as a group of more than 80 species and subspecies at three ploidy levels, i.e., A- or C-genome diploids, AB- or AC-genome tetraploids and ACD-genome hexaploids. *Avena sativa* ($2n = 6\times = 42$), previously considered as a "minor cereal" and a high quality feed for horses and other farm animals, has been recently re-evaluated for human consumption on the basis of its nutritional value (Peterson 1992; Redaelli et al. 2005). Actually, compared to other cereals, oat is rich in protein (15–20 %), fat (5–9 %) and fiber (12–14 %), including, lignin, cellulose, hemicellulose and chiefly β-D-glucan, which, in humans, is involved in the natural defense against infections caused by viruses, bacteria and fungi, and in reduction of glucose and cholesterol levels in the

blood (Rondanelli et al. 2009). At present, naked oats are considered as the eligible raw material for human consumption thanks to the development of new genotypes characterized by earliness and high yield, as well as by superior quality of their end products, i.e., flakes, biscuits and bread. Moreover, naked cultivars are suitable for extraction of molecules of pharmaceutical and cosmetic use (Valentine 1995; Peterson 2004). Among the cereal crops, *A. sativa* is characterized by an extremely soft endosperm texture much softer than those measured in common wheat, rye, barley and einkorn wheat (Li et al. 1996; Pedersen et al. 1996; Pogna et al. 2002; Hansen et al. 2004; Iwami et al. 2005; Taddei et al. 2009). Kernel texture is a trait of great technological and trade importance due to its strong influence on a bulk of quality traits including flour yield, flour granularity, starch damage, water absorption, dough rheological properties, bread volume and crumb structure (Lindahl and Eliasson 1992; Dexter et al. 1994). In oats, the high kernel softness leads also to some negative agronomic traits especially in naked cultivars, because it determines, a high incidence of damaged caryopses during mechanical harvesting and threshing, which significantly reduces germination percentage as well as grain vitality and healthiness (Valentine and Hale 1990; Peltonen-Sainio et al. 2001). Moreover, from the technological point of view, a high number of broken kernels negatively affect size and quality of oat flakes. Therefore, comprehension of the molecular mechanisms underlying kernel texture in extra-soft *Avena* may help develop novel strategies to modulate this important genetic trait in cereal crops.

In common wheat, kernel texture is largely determined by the *Pina-D1* and *Pinb-D1* genes on chromosome 5DS coding for puroindolines A (PIN-A) and B (PIN-B), respectively. Puroindolines are two 13 kDa basic isoforms, 59.5 % similar in amino acid sequence, with a relative high number of cysteine and lysine residues. These proteins share a characteristic tryptophan-rich domain (TRD), which makes them unique among plant proteins; in particular, variation in endosperm texture was correlated with the affinity of the TRD for the polar lipids of the amyloplast. Moreover, puroindolines share a highly conserved skeleton of 10 cysteine residues (Blochet et al. 1993) typical of the 2S protein superfamily (Egorov et al. 1996).

The complete sequences of genes coding for PIN-A and PIN-B have been described in the *Triticum/Aegilops* taxa, rye and barley including the A, D, C, H, M, N, R, S, and U genomes (Gautier et al. 2000; Lillemo et al. 2002; Massa et al. 2004; Massa and Morris 2006; Chen et al. 2005; Taddei et al. 2009). However, the A and B genomes of tetraploid species *T. turgidum* spp. *durum* (durum wheat) and hexaploid species *T. aestivum* (common wheat) are devoid of puroindoline-encoding genes as a result of deletions caused by transposon excision immediately after the formation of durum wheat (Gautier et al. 2000; Chantret et al. 2005).

In *Avena* a study conducted by Tanchak et al. (1998) in immature seeds of the Canadian oat cv. Hinoat led to the identification of two pairs of cDNA clones, i.e., 3B3-5 plus 3B3-7 and 3B3T-3 plus 3B3T-5. The first pair encodes a 147-amino acid protein with a TRD containing four tryptophan residues, while 3B3T-3/3B3T-5 pair encodes a 142-amino acid protein with the same TRD. The high similarity between proteins encoded by these two pairs of genes suggests that they represent different members of the same gene family. In addition, the presence of the typical ten cysteine residue skeleton indicates that these proteins belong to the 2S family. Therefore, Tanchak et al. (1998) introduced the collective name of tryptophanins for puroindolines, 3B3/3B3T proteins and grain softness proteins (GSPs), this latter group of proteins showing 60 % similarity to PINs and a TRD consisting of two tryptophan residues (Rahman et al. 1994). Using primers specific for *Pina-D1* and *Pinb-D1*, Gautier et al. (2000) revealed two oat genes coding for avenoindoline-A (AIN-A) and avenoindoline-B (AIN-B). In particular, the deduced amino acid sequence of AIN-A resulted to be identical to that of PIN-A except for the N81 V replacement. On the other hand, AIN-B and PIN-B showed 91 % similarity in their sequences. A monoclonal antiserum specific for friabilin, a protein group largely represented by PIN-A and PIN-B, was found to strongly react with proteins associated with oat starch granules. These proteins were assumed to be 3B3/3B3T, even though their sequence similarity with pur oindolines/avenoindolines is as low as 48–61 % (Mohammadi et al. 2007).

The present work reports the results of biochemical and molecular characterization of oat endosperm indolines and their encoding genes in *Avena* species at different ploidy levels. The oat indolines identified in this study proved to be specific of the *Avena* genus and therefore the comparison of the sequences of their encoding genes in different ploidy level species could contribute to clarify the genetic relationship and the genome evolution in *Avena* ssp. Furthermore, transgenic durum wheat genotypes were developed to probe the role played by oat indolines in determining kernel texture.

Materials and methods

Plant material

Seeds of 17 oat cultivars (Abel, Adam, Bikini, Bullion, Donata, Flavia, Grafton, Harpoon, Ikon, Konradine, Kripton, Kinon, Lisbeth, Primula, Terra, Tropicale, Vir 2301), grown at Montelibretti (Rome) in randomized blocks with

three replications, were used in the present study. The elementary plot of 10 m^2 consisted of eight rows, 17 cm apart, sown with 400 germinating kernels/m^2. Common wheat cvs Chinese Spring and Bolero were analyzed as well. Wild oats *A. atlantica, A. barbata, A. canariensis, A. clauda, A. eriantha, A. hirtula, A. insularis,* and *A. strigosa,* from the CRA-MAC (Bergamo, Italy) collection, were used as well. Seeds of *A. sativa* cvs Donata and Primula were harvested at 7, 14, 21, 28 and 35 days post anthesis (DPA), immediately frozen in liquid nitrogen and stored at −80 °C until used for RNA and protein extraction.

Kernel hardness

Kernel hardness was evaluated on 300 manually de-hulled kernels by the Perten SKCS 4100 (Springfield, IL, USA) following the manufacturer's operating procedure. The instrument was set in a range of hardness between −40 and +120.

Protein extraction and electrophoretic analysis

Air-dried starch granules were obtained as described by Corona et al. (2001). Starch granules were suspended (1:6 w/v) in a solution containing 50 mM NaCl and 50 % (v/v) propan-2-ol. After sonication for a few seconds, the suspension was kept in agitation at room temperature for 1 h and then centrifuged at 8,000 × *g* for 10 min. Proteins in the supernatant were precipitated with two volumes of acetone at −20 °C overnight and vacuum dried. Starch-bound proteins were reduced and fractionated by SDS-PAGE as described previously (Gazza et al. 2005). In addition, proteins were reduced and alkylated by incubation for 30 min at 60 °C in 100 μl of a solution made of 4.5 mg of DTT in 1.5 ml of 50 % propan-2-ol and 163 mM Tris–HCl pH 8.8. After centrifugation, the supernatant was transferred into a new tube, diluted with the same volume of a solution containing 50 % (v/v) propan-2-ol, 0.08 M Tris–HCl, pH 8.5, 0.04 M vinylpyridine and incubated for 30 min at 37 °C. Alkylated proteins were fractionated by A-PAGE and A-PAGE × SDS-PAGE (Gazza et al. 2006). Protein extractions and electrophoretic fractionations were performed at least twice for each genotype.

Western blotting

Western blotting was performed in a Biorad (USA) Semi Dry Transfer Cell using nitrocellulose membranes (Hybond-C Extra, GE Healthcare,USA). SDS-PAGE and A-PAGE × SDS-PAGE gels were equilibrated for 30 min in a transfer buffer, pH 9.2, containing 80 mM Tris, 13 mM glycine and 20 % (v/v) methanol in distilled water, transferred between two double layers of 3 MM chromatography paper (GE Healthcare), pre-equilibrated in the same transfer buffer, and blotted at 23 V for 1 h. A-PAGE gels were equilibrated and transferred as described by Gazza et al. (2006). After the transfer, membranes were maintained for 1 h in PBS buffer containing 5 % (w/v) blocking agent (GE Healthcare), and incubated for 16 h in the same buffer containing 0.2 % (w/v) blocking agent and a 1:500 dilution of a polyclonal specific antiserum prepared by standard methods (Primm Srl, Milan, Italy). The polyclonal antisera were developed in rabbits immunized with (a) synthetic peptide DRASKVIQEAKNLPP homologous to 116–131 region of AIN-A and PIN-A (b) synthetic peptide LGGFF-GTQQGLIGKR homologous to 106–120 region of 3B3-7 or (c) synthetic peptide LGGFLGFQQSEIMKQ homologous to 102–116 region of 3B3T-5. After three washes with PBS buffer and incubation for 4 h with a 1:2,500 dilution of goat anti-rabbit horseradish peroxidase conjugate (Promega, USA), blots were stained in a solution containing 50 ml PBS buffer, 10 ml methanol, 30 mg 4-chloro-1-naphthol and 1 ml H_2O_2 (30 %). Peroxidase activity was also visualized by chemiluminescence using the Supersignal Detection Kit (Pierce, USA), following the manufacturer's instructions. Western blotting was performed at least twice for each genotype.

DNA and RNA extraction, RT-PCR and PCR amplification

Genomic DNA was isolated from young leaves using cetyltrimethylammonium bromide (CTAB) method (Ausubel et al. 1994). Total RNA was extracted with TRIZOL Reagent (Invitrogen, USA) from 70 mg of seeds collected at 7, 14, 21, 28 and 35 DPA. First-strand cDNA synthesis from 750 ng of total RNA was performed with the ImPromII Reverse Transcription System (Promega, USA) according to manufacturer's instructions in 20 μl final volume. An aliquot (5 μl) of cDNA was used as template for PCR reactions (50 μl final volume) using 2.5 U of Go Taq DNA polymerase (Promega, USA); PCR primers (50 pmol each reaction) used in this study are listed in Table 1. The amplicon obtained with the primer pair PP6 (Table 1) specific to the oat β-tubulin housekeeping gene (GenBank accession number X54852.1) was used as a loading control. The samples, denaturated at 94 °C for 3 min before the addition of Taq, were submitted to 35 cycles of 1 min of denaturation at 94 °C, 1 min annealing at T_m (Table 1) and 1.5 min elongation at 72 °C. A final cycle with an extension of 7 min at 72 °C completed the reaction. PCR products were analyzed on 1.8 % agarose gels, stained with ethidium bromide and visualized under UV. Gels were analyzed with the UVIpro Bronze Gel Documentation System (Uvitec, UK), using the UVIpro 12.5 software.

PCR amplifications were performed in 50 μl containing 50 ng of genomic DNA, 10 pmol of each primers (Table 1),

Table 1 Primer pairs and annealing temperatures used for PCR amplification

Name	Forward primer (5′-3′)	Reverse primer (5′-3′)	T_m (°C)	Amplicon size (bp)	Amplified gene
PP1	ATGAAGGCCCTCTTCCTCA	TCACCAGTAATAGCCAATAGTG	58	447	*Aina*
PP2	ATGAAGACCTTATTCCTCC	TCACCAGTAATAGCCACTAGG	54	447	*Ainb*
PP3	GAAACATGAAGACCTTACTGC	CCAGTAGTAGCCAGTAGTAAG	58	441	*Vin-1*
PP4	GCTTCTATTCATCCTACCACC	TCACATTTAGTGGTATGCGAC	60	528	*Vin-2*
PP5	ATGAAGGCCTTATTCCTCCTAGCTT	GTAATATCCAAGATTGGTAGGGAAG	58	426	*Vin-3*
PP6	ATGGACCTTGAGCCCGGT	CTGATACTGTGGTTGAGCC	[a]	349	*β-tubulin*

[a] T_m for the β-tubulin primer pair corresponds to that of co-amplified gene

100 μM of each dNTP and 2.5 U of Jump Start REDAccu Taq LA DNA Polymerase (SIGMA). The samples were denaturated at 94 °C for 3 min and submitted to 35 cycles of 1 min at 94 °C, 1.5 min at T_m and 1.5 min at 72 °C; a final cycle with an extension of 7 min at 72 °C completed the reaction.

DNA cloning and sequencing

Amplification products were eluted from agarose gels with the Nucleospin-Extract kit (MachereyNagel, Germany), cloned as previously described (Gazza et al. 2006) and sequenced with the Beckman Coulter CEQ 8000 sequencer according to the manufacturer's instructions. DNA sequences were deposited in the GenBank sequence database at NCBI. DNA amplification, cloning and sequencing were performed at least three times for each genotype.

RACE (rapid amplification cDNA ends)

An aliquot (10 μg) of total RNA extracted from seeds harvested at 28 DPA was submitted to 3′ and 5′ RACE experiments performed with 3′ and 5′ RACE System kits (Invitrogen, USA) according to manufacturer's instructions. Primers used for cDNA extension are shown in Table 1. cDNA samples, denaturated at 94 °C for 3 min before the addition of 2.5U of Go Taq DNA polymerase (Promega, USA), were submitted to 35 cycles of 1 min of denaturation at 94 °C, 1 min annealing at T_m and 1.5 min elongation at 72 °C, with final extension of 7 min at 72 °C. RACE experiments were performed at least twice for each genotype.

In-gel digestion and mass spectrometric analysis

Selected protein spots from the 2-DE gel were excised manually and transferred into 1.5 ml microcentrifuge tubes. Low-abundance protein spots were removed from all the replicate gels and pooled. The spots were washed and subjected to in-gel trypsin digestion according to Shevchenko et al. (1996). After soaking trypsin (Modified porcine trypsin, Promega,) into the gel pieces, the supernatant containing excess trypsin was removed and the gel pieces were covered with 50 μl of 50 mM NH_4HCO_3 and incubated at 37 °C overnight. The enzymatic reaction was stopped by cooling the gel pieces and the supernatant solution at −20 °C. After in-gel digestion, the solution of peptides was transferred into clean 0.5-ml tubes. The peptides were extracted from gel pieces with 40 μl 0.1 % TFA and subsequently with the same volume of acetonitrile. This extraction procedure was repeated three times. The total extracts were pooled, combined with the first supernatant, lyophilized and dissolved in 15 μl of 0.1 % TFA. Capillary RP-HPLC/nESI-MS/MS was performed using an Ultimate 3000 LC system combined with an autosampler and a flow splitter 1:100 (Dionex Corporation, USA) coupled online with a linear ion trap nano-electrospray mass spectrometer (LTQ, Thermo Fischer Scientific, USA). Ionization was performed with liquid junction using a noncoated capillary probe (30 ± 2 μm i.d.; New Objective, USA).

The peptides solution was directly loaded onto a C18 μ-pre-column cartridge (0.3 × 5 mm, 100 Å, 5 μm, PepMap, Dionex) equilibrated with 0.5 % aqueous formic acid (FA) at a flow rate of 20 μl/min for 4 min. Subsequently, peptides were applied onto a C18 capillary column (0.18 × 150 mm, 300 Å, 5 μm, Thermo Electron) and eluted at room temperature with a linear gradient of CH_3CN–0.5 % FA/H_2O–0.5 % FA from 10–50 % in 50 min at a flow rate of 2 μl/min. HPLC-grade water and CH_3CN were provided by Carlo Erba (Milan, Italy).

Repetitive mass spectra were scanned using the following electrospray ion source parameters: capillary temperature, 220 °C; spray voltage, 1.9 kV. Peptide ions were analyzed by the data-dependent method as follows: (a) full MS scan (mass-to-charge ratio 350–2,000); (b) ZoomScan (scans three major ions with higher resolution; isolation width 2 Da); and (c) MS/MS of the 3 major ions (Q 0.250, collision energy 29 a.u.). Mass calibration was made using a standard mixture of caffeine (Mr 194.1 Da), MRFA peptide (Mr 423.6 Da) and Ultramark (Mr 1621 Da). Data acquisition was performed using the Excalibur v. 1.4 software (ThermoFisher Scientific).

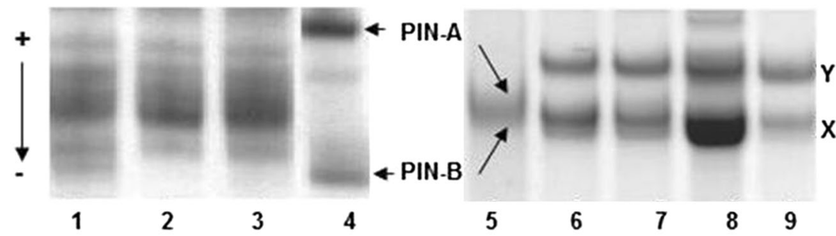

Fig. 1 A-PAGE fractionation of starch-bound proteins. *Lanes 1–4* Native proteins from oat cvs Boa Fè, Corbit, and Algeribee, and common wheat cv. Chinese Spring. *Lanes 5–9* Reduced and alkylated proteins from common wheat cv. Chinese Spring, oat cvs Boa Fè, Corbit, Algeribee and *Avena strigosa*. *PIN-A* puroindoline A, *PIN-B* puroindoline B, *X* fast-moving alkylated proteins, *Y* slow-moving alkylated proteins

Database search and protein identification

MS/MS data were used to perform protein identifications by searching in a non-redundant protein sequence database (NCBInr) using the MOWSE algorithm as implemented in the Mascot search engine version 2.3 (Matrix Science: http://www.matrixscience.com). The following parameters were used for database searches: taxonomy, *Viridiplantae*; cleavage specificity, trypsin with 2 missed cleavages allowed; mass tolerance of 1.2 Da for the precursor ions and a tolerance of 0.6 Da for the fragment ions; allowed modifications, Cys pyridylethyl (fixed), oxidation of Met (variable), transformation of *N*-terminal Gln and *N*-terminal Glu residue in the pyroglutamic acid form (variable). All identifications obtained by means of the MASCOT software were confirmed by manual interpretation of the MS/MS spectra assisted by The General Protein/Mass Analysis for Windows software (GPMAW; http://welcome.to/gpmaw) and the PepNovo software (http://proteomics.ucsd.edu/Software/PepNovo.html) to confirm or disregard peptide sequence assignation and therefore protein identification. Interpretation of unassigned MS/MS spectra was performed manually.

Durum wheat transformation

Two transformation vectors were assembled by the insertion of the coding region of either gene *Vin-D2a* (JQ518369) or gene *Vin-A3a* (JQ518372) in the *Sal*I and *Xba*I sites of the pRDPT plasmid (Tosi et al. 2004) between the endosperm promoter and terminator of the Dx5 HMW-glutenin gene (*Glu-D1-1a*) (Anderson et al. 1989) (Online Resource 1). The *Sal*I and *Xba*I sites flanking the coding region of *Vin-D2a* or *Vin-A3a* genes were generated by PCR amplification in a final volume of 50 µl with 300 ng of genomic DNA of the oat cv. Donata, 2.5 U of High Fidelity Taq (Promega), 1× Taq buffer, 10 pmol of each primer (PP4 or PP5, Table 1) and 100 µM of each dNTP. The amplification conditions were an initial cycle at 94 °C for 1 min and 35 cycles at 94 °C for 1 min, 58 °C for 1.5 min and 72 °C for 1.5 min. The reaction was ended with a final cycle at 72 °C for 10 min. After digestion with *Sal*I and *Xba*I restriction enzymes (New England, USA), either *Vin-D2a* or *Vin-A3a* was inserted into pRDPT generating a 5,660-bp plasmid (Online Resource 1). The pAHC20 construct carrying the *bar* gene coding for resistance to phosphinothricin was used as the selectable marker. For co-transformation experiments, 1,863 embryo-derived calli of *Triticum durum* cv. Svevo were co-bombarded with pRDPT + *Vin-D2a*, pRDPT + *Vin-A3a* and pAHC20 in 1:1:6 molar ratio as described previously (Janni et al. 2008). The presence of pRDPT + *Vin-D2a* and pRDPT + *Vin-A3a* in plants resistant to bialaphos (phosphinothricin-alanyl-alanine) and in their progeny was verified by PCR. Bialaphos-resistant plants and plants of control cv. Svevo were grown in pots in a growth chamber under a temperature regimen of 24 °C day/17 °C night with lighting from sodium lamps to maintain a day length of 16 h. T_0 and T_1 plants were allowed to set seeds, and seeds were harvested from individual plants.

Results

A-PAGE fractionation of starch granule proteins in oat cultivars

Upon electrophoretic fractionation by A-PAGE at pH 3.1, native puroindoline A (PIN-A) and puroindoline B (PIN-B) extracted from common wheat cv. Chinese Spring appeared as two prominent bands (Fig. 1, lane 4), whereas proteins extracted from the starch granules of *A. sativa* occurred as several bands with electrophoretic mobilities intermediate between PIN-A and PIN-B (Fig. 1, lanes 1–3). Reduction and alkylation of these proteins resulted in quite different A-PAGE patterns (Fig. 1, lanes 5–9). In particular, proteins from oat cultivars separated into two broad bands marked X and Y, whereas PIN-A and PIN-B co-migrated as a single diffuse band with an electrophoretic mobility comparable to that of X-type proteins. It is noteworthy that X- and Y-type proteins were also found in the A-PAGE

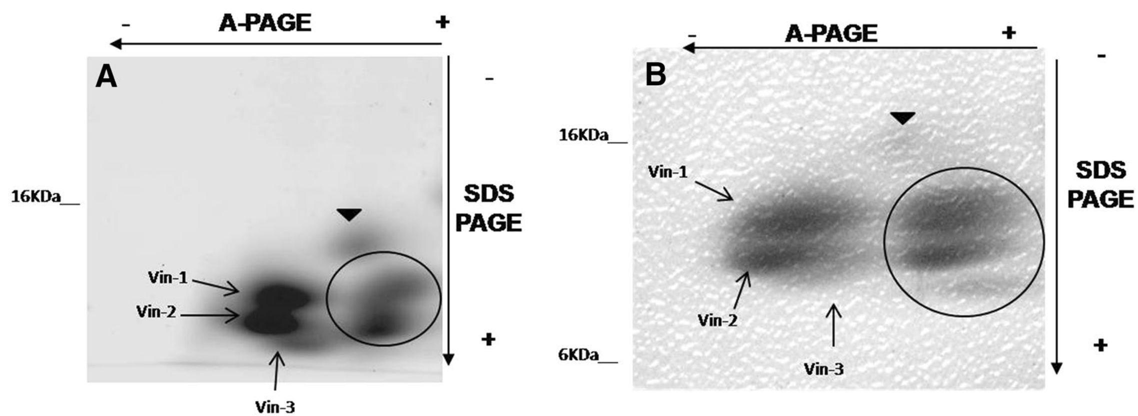

Fig. 2 Two-dimensional A-PAGE × SDS-PAGE fractionation of starch-bound proteins from **a** *Avena sativa* cv Donata and **b** *Avena strigosa*. Vin = Vromindoline. *Circled spots* are Avena-α-Amylase-Trypsin-Inhibitors (AATI). The *black arrowhead* indicates a high-molecular-weight protein

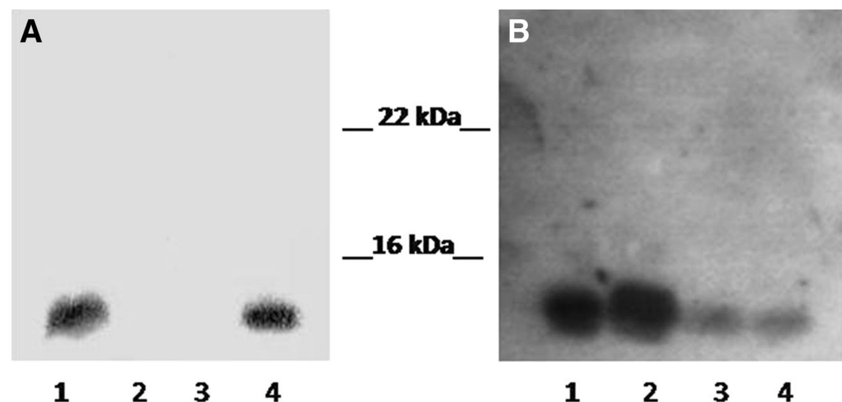

Fig. 3 Reaction of the polyclonal antiserum specific to PIN-A and AIN-A against starch-bound proteins fractionated by SDS-PAGE. **a** Cloronaphtol assay: *1*, common wheat cv. Chinese Spring; *2* and *3*, oat cvs Primula and Donata; *4*, common wheat cv. Bolero. **b** Chemo-luminescent assay (*1* and *2*) common wheat cvs Chinese Spring and Bolero (*3* and *4*) oat cvs Primula and Donata

fractionations of starch-bound proteins from A-genome *A. atlantica, A. canariensis, A. hirtula* and *A. strigosa* (Fig. 1, lane 9), C-genome *A. clauda* and *A. eriantha*, and tetraploid species *A. barbata* (genome AB) and *A. insularis* (genome AC) (data not shown).

A-PAGE × SDS-PAGE fractionation of starch granule proteins in oat species

When fractionated by two-dimensional A-PAGE × SDS-PAGE, both X-type and Y-type proteins in hexaploid oats split into three spots in the 6–15 kDa region of the gel (Fig. 2a). The X-type components were denominated vromindolines (VIN), *vromi* being the Greek name for oats. In particular, VIN-2 showed an apparent molecular weight intermediate between VIN-1 and VIN-3. This latter polypeptide occurred as a faint spot in all oat cultivars analyzed.

Furthermore, the two-dimensional fractionation of diploid or tetraploid *Avena* species mentioned above showed the same pattern of three X-type spots (Fig. 2b). The Y-type spots, named Avena-α-Amylase-Trypsin-Inhibitors (AATI), and an additional spot of high molecular weight (Fig. 2, arrowhead), will be discussed in a separated paper.

Identification of vromindolines by Western blotting and mass spectrometry

The colorimetric assay with cloro-naphthol revealed that the polyclonal antiserum specific to the DRASKVIQEAKNLPP sequence present in both wheat PIN-A and oat AIN-A strongly reacts with the starch-bound proteins extracted from common wheat and fractionated by SDS-PAGE (Fig. 3a, lanes 1 and 4). Surprisingly, starch-bound proteins extracted from hexaploid oats did not show any reaction with this antibody (Fig. 3a, lanes 2 and 3). However, the luminol-dependent chemo-luminescence assay, which is ten thousand more sensitive than the colorimetric assay based on cloro-naphthol, revealed a weak reaction of oat proteins (Fig. 3b, lanes 3 and 4) compared with those extracted from common wheat cvs Chinese Spring and Bolero (Fig. 3b, lanes 1 and 2), suggesting that AIN-A is a minor component of proteins associated with

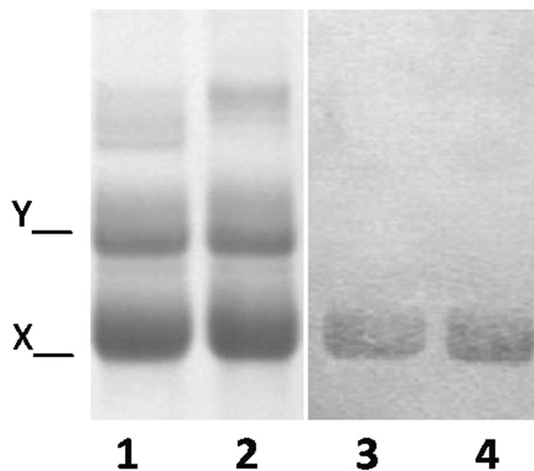

Fig. 4 A-PAGE fractionation of starch-bound proteins (*left side*) and immunostaining with the polyclonal antiserum specific to protein 3B3-7 (*right side*). *1*, *3* oat cv. Donata; *2*, *4* oat cv. Primula

oat starch granules. Alkylated VIN proteins fractionated by A-PAGE strongly reacted with the specific antisera developed against 3B3 (Fig. 4) or 3B3T. In particular, anti-3B3 was found to react with VIN-2 in the A-PAGE × SDS-PAGE pattern of hexaploid oats (Online Resource 2B), whereas anti-3B3T reacted with VIN-3 (data not shown). The same results were obtained in the diploid and tetraploid oat species analyzed here. By contrast, anti-3B3 and anti-3B3T antisera gave no reaction with starch-bound proteins extracted from common wheat.

The peptides obtained by trypsin digestion of VIN-2 were analyzed by RP-HPLC/nESI-MS/MS and found to correspond with approximately 78 % of mature 3B3 protein (Table 2, underlined sequences). The presence of the 9-mer ^{29}EEVEGPHDR sequence in this peptide pool suggested that mature 3B3 starts with E29 and originates from post-translational cleavage of a 19-mer signal peptide plus an additional 9-mer sequence. Furthermore, identification of molecular ions with the P to A or P to S substitution in ^{29}EEVEG<u>P</u>HDR indicated that VIN-2 includes at least three different polypeptides. Peptide ions with either H or R at position 104 of VIN-2 were observed as well (Table 2).

Trypsin-digested VIN-3 released peptide fragments covering about 45 % of mature 3B3T protein (Table 2, underlined sequences), whereas digestion of VIN-1 produced several peptides absent in VIN-2 and VIN-3, along with the DFPLTWPWKWWK sequence that harbors the WPWKWWK motif typical of vromindolines (Table 2, underlined sequences). All VIN-1 peptide ions were found to be encoded by the novel *Vin-A1a* gene (GenBank accession JQ518366) obtained by 3′- and 5′-RACE of mRNA extracted from immature seeds of oat cv. Donata. This aspect will be discussed later. Furthermore, digestion of VIN-1 and subsequent MS/MS analysis allowed the identification of a single amino acid substitution at position 52 (T or M) (Table 2), indicating the polymorphic nature of VIN-1. Finally, no peptides belonging to avenoindolines AIN-A or AIN-B were found in any of the trypsin-digested VIN spots.

Vromindoline mRNA synthesis and VIN deposition on starch granules in developing kernels

During the grain-filling stage, oat cvs Primula and Donata produced transcripts coding for VIN-2 and VIN-3, as determined by RT-PCR amplification. Synthesis of VIN-1 transcripts was not investigated. In particular, VIN-2 and VIN-3 transcripts reached a peak of accumulation at 21 DPA (Online Resource 3A), although mRNAs coding for VIN-2 were detectable as early as 7 DPA, 7 days before those coding for VIN-3 (Online Resource 3B). By contrast, AIN-A mRNAs occurred in trace amounts in immature kernels at 7–21 DPA, and slightly increased at 28 DPA (Online Resource 3C). No AIN-A transcripts were detectable at 35 DPA. Notably, mRNAs coding for AIN-B were not found in any of the time points analyzed.

The starch granules of immature kernels collected at 14 DPA from oat cvs Primula and Donata revealed the first traces of vromindolines (Online Resource 4A, lane 2). Deposition of VIN proteins on the starch granules reached its maximum at 28 DPA and slightly decreased at maturity (42 DPA). VIN-2 and VIN-3 followed the same pattern of accumulation, as determined by their immune reactions with anti-3B3 and anti-3B3T antisera, respectively (Online Resource 4B). By contrast, neither developing nor mature kernels showed immune reactions with the anti-PIN-A antiserum, as revealed by the cloro-naphthol assay.

Genes coding for avenoindoline-A

PCR amplification of genomic DNA from single plants of oat cvs Donata and Primula using primers specific to wild-type allele *Pina-D1a* (PP1, Table 1) produced a 447-bp amplicon, which was used to develop recombinant *E. coli* clones. Sequencing of these clones revealed two genes provisionally called *Aina-A1a* (GenBank JX096490) and *Aina-C1a* (GenBank JX096491) in each cultivar. Avenoindoline-A1 (AIN-A1) encoded by *Aina-A1a* and avenoindoline-A2 (AIN-A2) encoded by *Aina-C1a* differ from each other for two amino acid substitutions at positions 26 and 87 (Table 3). A-genome species *A. hirtula* and *A. strigosa* exhibited a single gene coding for avenoindoline-A1. In particular, synonymous codons GGT, GGG and GGA at position 22–24 of the DNA sequence coding for the signal peptide were found to be the only difference among genes *Aina-A^{h}1a* (GenBank JX096499) in *A. hirtula*, *Aina-A^{s}1a*

Table 2 Amino acid sequences of vromindolines in *A. sativa* and other oat species

Protein	Gene	Species	Amino acid sequence							
Vromindoline 1										
VIN-1.1	*Vin-A1a*	*A. sativa*	MKTLLLLALL^{10}	ALAASTAFAQ^{20}	YAQDDGWNEQ ^{30}	GGEATGCEQQ^{40}	QANLDSCKDY^{50}	VTERCFT-MKD^{60}	FPLTWP-WKWW^{70}	KGGCE-HEVRY^{80}
	Vin-A^{h}1a	*A. hirtula*	- - - - - - - - L-	- - - - - - - - - -	- A - DD - - - - -	- - - - T - - - - -	- - N - - - - - - -	- T - - - - - - - -	- - - - - - - - - -	- - - - - - E - - -
	Vin-A^{s}1a	*A.strigosa*	- - - - - - - - L-	- - - - - - - - - -	- A - DN - - - - -	- - - - T - - - - -	- - N - - - - - - -	- T - - - - - - - -	- - - - - - - - - -	- - - - - - E - - -
VIN-1.2	*Vin-C1a*	*A. sativa*	- - - - - - - - -V-	- - - - - - - - - -	- D - VD - - - - -	- - - - A - - - - -	- - K - - - - - - -	- L - - - - - - - -	- - - - - - - - - -	- - - - - - E - - -
	Vin-C^{c}1a	*A. claude*	–	- - - - - - - - - -	- D - DD - - - - -	- - - - A - - - - - Q	- - N - - - - - - -	- L - - - - - - - -	- - - - - - - - - -	- - - - - - E - - -
VIN-1.3	*Vin-D1a*	*A. sativa*	- - - - - - - - L-	- - - - - - - - - -	- A- DD - - - - -	- - - - T - - - - -	- - N - - - - - DY	VM ERCFT-MKD	FPLTWP-WKWW^{70}	K - - - - - K - - -
VIN-1.1	*Vin-A1a*	*A. sativa*	QCCEQLNQVS^{90}	QQCRCK AIWR^{100}	AVEHELGGFL^{110}	GLQKGEIGKR^{120}	LLRAK SIPSK^{130}	CNMGPQC-NFP^{140}	LTTGYYW^{147}	
	Vin-A^{h}1a	*A. hirtula*	- - - - - - - - - -	- - - - - - - - W -	- - - - - - - - - -	- - - K - - - - - R	- - - - - - - - S -	- - - A - - - - - -	- - - - - - -	
	Vin-A^{s}1a	*A.strigosa*	- - - - - - - - - -	- - - - - - - - W -	- - - - - - - - - -	- - - K - - - - - R	- - - - - - - - S -	- - - G - - - - - -	- - - - - - -	
VIN-1.2	*Vin-C1a*	*A. sativa*	- - - - - - - - - -	- - - - - - - - W -	- - - - - - - - - -	- - - K - - - - - R	- - - - - - - - S -	- - - G - - - - - -	- - - - - - -	
	Vin-C^{c}1a	*A. claude*	- - - - - - - - - -	- - - - - - - - F -	- - - - - - - - - -	- - - Q - - - - - R	- - - - - - - - A -	- - - G - - - - - -	- - - - -	
VIN-1.3	*Vin-D1a*	*A. sativa*	- - - - - - - - - -	- - - - - - - - W -	- - - - - - - - - -	- - - K - - - - - H	LLR - - - - - S -	- - - G - - - - - -	- - - - - - -	
Vromindoline 2										
VIN-2.1	*Vin-A2a*	*A. sativa*	MKIFFFLALL^{10}	ALVVSATFAQ^{20}	YVESDGSYEE^{30}	VEGPHDRCQQ^{40}	HQMKLDSCRE^{50}	YVAER CTTMR^{60}	DFPITWP-WKW^{70}	WKGG-CEEVRN^{80}
	Vin-A^{h}2a	*A.hirtula*	- - - - — - - -	- - - - - - - - - -	- V- - - - - - - -	- - - PH - - - - -	- - - - - - - - - -	- - - ER - - - - -	- - - - - - P - - -	- - - - - - - V - -
	Vin-A^{s}2a	*A.strigosa*	- - - - - - - - - -	- - - - - - - - - -	- V- - - - - - - -	- - - PH - - - - -	- - - - - - - - - -	- - - ER - - - - -	- - - - - - S - - -	- - - - - - - V - -
	Vin-A^{i}2a	*A. insularis*	- - - - - - - - - -	- - - - - - - - - -	- V - - - - - - - -	- - - PH - - - - -	- - - - - - - - - -	- - - ER - - - - -	- - - - - - P - - -	- - - - - - - V - -
VIN-2.2	*Vin-C2a*	*A. sativa*	- - - - - - - - - -	- - - - - - - - - -	- A- - - - - - EE	VEGSHDRCQQ	HQMK- - - - - -	- - - ER - - - - -	- - - - - - P - - -	- - - - - - - L- -
	Vin-C^{e}2a	*A. eriantha*			- - -	- - - SH - - - - -	- - - - - - - - - -	- - - ER - - - - -	- - - - - - P - - -	- - - - - - - L - -
	Vin-C^{i}2a	*A. insularis*	- - - - - - - - - -	- - - - - - - - - -	- A - - - - - - - -	- - - SH - - - - -	- - - - - - - - - -	- - - ER - - - - -	- - - - - - P - - -	- - - - - - - L - -
VIN-2.3	*Vin-D2a*	*A. sativa*	- - - - - - - - - -	- - - - - - - - - -	- V- - - - - - EE	VEGAH DRCQQ	HQMK- - - - - -	- - - ER - - - - -	- - - - - - P - - -	- - GGCEEVRN
VIN-2	*3B3-5*	*A. sativa*	- - - - - - - - - -	- - - - - - - - - -	- V- - - - - - - -	- - - PA - - - - -	- - - - - - - - - -	- - - DG - - - - -	- - - - - - P - - -	- - - - - - - V - -
	3B3-7	*A. sativa*	- - - - - - - - - -	- - - - - - - - - -	- V- - - - - - - -	- - - PH - - - - -	- - - - - - - - - -	- - - DG - - - - -	- - - - - - P - - -	- - - - - - - V - -
VIN-2.1	*Vin-A2a*	*A. sativa*	ECCQLLGQMP^{90}	SECRCDAIWR^{100}	SIQHELGGFF^{110}	GTQQGLIGKR^{120}	LKIAKSLPTQ^{130}	CNMGPEC-NIP^{140}	VTFGYYW^{147}	
	Vin-A^{h}2a	*A.hirtula*	- - - - - - - - - -	S - - - - - - - - -	- -QH - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- T F - - - -	
	Vin-A^{s}2a	*A.strigosa*	- - - - - - - - - -	S - - - - - - - - -	- -QH - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- T F - - - -	
	Vin-A^{i}2a	*A. insularis*	- - - - - - - - - -	S - - - - - - - - -	- -QH - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- T F - - - -	
VIN-2.2	*Vin-C2a*	*A. sativa*	ECCQLLGQMP	SECRCDAIWR	SIQRELGGFF	GTQQGLIGKR	- - - - - - - - - -	- - - - - - - - - -	- T M - - - -	
	Vin-C^{e}2a	*A. eriantha*	- - - - - - - - - -	S - - - - - - - - -	- -RR - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- NM- - - -	
	Vin-C^{i}2a	*A. insularis*	- - - - - - - - - -	S - - - - - - - - -	- -QR- - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- T M - - - -	
VIN-2.3	*Vin-D2a*	*A. sativa*	ECCQLLGQMP	SECR - - - - - -	- -QH - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- T F - - - -	

Table 2 continued

Protein	Gene	Species	Amino acid sequence							
VIN-2	*3B3-5*	*A. sativa*	- - - - - - - - - -	S - - - - - - - - -	- -QH - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- T F - - - -	
	3B3-7	*A. sativa*	- - - - - - - - - -	W- - - - - - - - -	- -QH - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- T F - - - -	
Vromindoline 3										
VIN-3.1	*Vin-A3a*	*A. sativa*	MKALFLLAFL^{10}	ALAASAAFAQ^{20}	QYADTGVGGW^{30}	DGCMPEK ARL^{40}	NSCK DYVVER^{50}	CLTLK DIPIT^{60}	WPWKWWK- GGC^{70}	ESEVR GQCCM^{80}
	Vin-A^{c}3a	*A. canarien-sis*	- - - - - - - AL-	- L - - - - - F - -	- - - D- GV - - -	- - - - - - - - - -	- - - K - - - - - -	- - - - -D- – -	- - - K - - - - - -	- - - - G - - - -
	Vin-A^{s}3a	*A.strigosa*	- - - - - - - LA-	- A - - - - - S - -	- - - D- GV - - -	- - - - - - - - - -	- - - R - - - - - -	- - - - -D- - - -	- - - K - - - - - -	- - - - G - - - -
	Vin-A^{i}3a	*A.insularis*	- - - - - - - AF-	- L - - - - - F - -	- - - D- GV - - -	- - - - - - - - - -	- - - K - - - - - -	- - - - -D- - - -	- - - K - - - - - -	- - - - S - - - -
	3B3T	*A. sativa*	- - - - - - - AF-	- L - - - - - F - -	- - - D- GV - - -	- - - - - - - - - -	- - - K - - - - - -	- - - - -D- - - -	- - - K - - - - - -	- - - - S - - - -
VIN-3.2	*Vin-C3a*	*A. sativa*	- - - - - - - AF-	-L - - - - - F - -	- - - N- RA - - -	- - - - - - - - - -	- - - K - - - - - -	- - - - -D- - - -	- - - K - - - - - -	- - - - G - - - -
	Vin-C3b	*A. sativa*	- - - - - - - AF-	- L - - - - - F - -	- - - D-GM - - -	- - - - - - - - - -	- - - K - - - - - -	- - - - -G- - - -	- - - R - - - - - -	- - - - G - - - -
	Vin-C^{i}3a	*A. insularis*	- - - - - - - AF-	- L - - - - - F - -	- - - N- RA - - -	- - - - - - - - - -	- - - K - - - - - -	- - - - -D- - - -	- - - K - - - - - -	- - - - G - - - -
VIN-3.1	*Vin-A3a*	*A. sativa*	ELNQIAPHCR^{90}	CKAIWR AVQG^{100}	ELGGFLGFQQ^{110}	SEIMKQ VHVA^{120}	QSLPSR CNMG^{130}	PNCNFP TNLG^{140}	YY^{142}	
	Vin-A^{c}3a	*A. canarien-sis*	- - - - - - - - - -	- - - - - - - V- -	- - - - - - - - - -	- - - - - - - - - -	- S- - - R - - - -	- - - - - - - - - -	- -	
	Vin-A^{s}1a	*A.strigosa*	- - - - - - - - - -	- - - - - - - V- -	- - - - - - - - - -	- - - - - - - - - -	- S –- K - - - -	- - - - - - - - - -	- -	
	Vin-A^{i}3a	*A. insularis*	- - - - - - - - - -	- - - - - - - V- -	- - - - - - - - - -	- - - - - - - - - -	- S- - - R - - - -	- - - - - - - - - -	- -	
	3B3T	*A. sativa*	- - - - - - - - - -	- - - - - - - V- -	- - - - - - - - - -	- - - - - - - - - -	- S- - - R - - - -	- - - - - - - - - -	- -	
VIN-3.2	*Vin-C3a*	*A. sativa*	- - - - - - - - - -	- - - - - - - V- -	- - - - - - - - - -	- - - - - - - - - -	- T –- K - - - -	- - - - - - - - - -	- -	
	Vin-C3b	*A. sativa*	- - - - - - - - - -	- - - - - - - I - -	- - - - - - - - - -	- - - - - - - - - -	- T –- K - - - -	- - - - - - - - - -	- -	
	Vin-C^{i}3a	*A. insularis*	- - - - - - - - - -	- - - - - - - V- -	- - - - - - - - - -	- - - - - - - - - -	- T- - - K - - - -	- - - - - - - - - -	- -	

Peptides identified by mass spectrometry are underlined

(GenBank JX096489) in *A. strigosa* and *Aina-A1a* in *A. sativa*, respectively. AC-genome *A. insularis* revealed allele *Aina-A*^{i}*1a* (GenBank JX096492) coding for an AIN-A1 protein identical to that encoded by *Aina-A1a*.

Genes coding for avenoindoline-B

The presence of genes coding for avenoindoline-B in hexaploid, tetraploid and diploid oat species was investigated by PCR amplification of genomic DNA from single plants using the primer pair specific for allele *Pinb-D1a* in common wheat (PP2, Table 1). In particular, *A. sativa* (cv. Primula), *A. strigosa* and *A. insularis*, the most likely tetraploid ancestor of the hexaploid oats (Ladizinsky 1998) gave the expected 447 bp amplicons, which were cloned in *E. coli*. Sequencing of five clones from *A. sativa* revealed three genes, *Ainb-A1a* (GenBank JX096494), *Ainb-C1a* (GenBank JX096495) and *Ainb-D1a* (GenBank JX096493) (Table 3). Avenoindoline B1 (AIN-B1) encoded by *Ainb-A1a* and avenoindoline B3 (AIN-B3) encoded by *Ainb-D1a* were found to differ from each other at positions 43 and 98, whereas avenoindoline B2 (AIN-B2) encoded by *Ainb-C1a* showed 13 amino acid substitutions with respect to both AIN-B1 and AIN-B3. A-genome *A. strigosa* exhibited allele *Ainb-A*^{s}*1a* (GenBank JX 096496) coding for a protein identical to AIN-B1 in *A. sativa*. On the other hand, ten clones obtained in AC-genome *A. insularis* were found to harbor two 447-bp-long genes, *Ainb-A*^{i}*1a* (GenBank JX096497) and *Ainb-C*^{i}*1a* (GenBank JX096498). This latter gene codes for an AIN-B2 protein identical to that encoded by *Ainb-C1a* in *A. sativa*, whereas *Ainb-A*^{i}*1a* codes for an AIN-B1 protein with the G43E substitution with respect to its counterpart encoded by *Ainb-A1a* in cv. Primula. Interestingly, this substitution occurs in AIN-B3 as well.

Genes coding for vromindolines

To assess the full genetic background of vromindolines, mRNA from immature kernels of cv. Donata collected at 28 DPA was amplified by 3′ RACE and 5′ RACE using the 5′-CTCACAGCCGCCCTTCCA-3′ primer designed on the conserved tryptophan domain of the *Pinb-D1a* gene and its reverse-complement 5′-TGGAAGGGCGGCTGTGAG-3′, respectively. This led to two amplicons, which were combined to form a Coding DNA Sequence (CDS) with 60 % similarity with allele *Pinb-D1a*. When amplified with primers positioned at the ends of this CDS (PP3, Table 1), single plants of cv. Donata produced 441-bp-long DNA fragments, which were cloned in *E. coli* and found to contain genes *Vin-A1a* (GenBank JQ518366), *Vin-C1a* (GenBank JQ518368) and *Vin-D1a* (GenBank JQ518367), coding for vromindoline-1.1 (VIN-1.1), vromindoline-1.2 (VIN-1.2) and vromindoline-1.3 (VIN-1.3), respectively (Table 2). These polypeptides differ from each other by 6 amino acid residues and show 50–58 % sequence similarity with 3B3 and 3B3T, respectively. Alignments of DNA and putative VIN-1 sequences obtained in diploid oat species revealed some variations with respect to their counterparts in *A. sativa*. In particular, allele *Vin-A*^{h}*1a* (GenBank JQ679296) in *A. hirtula* and allele *Vin-A*^{s}*1a* (GenBank JQ679297) in *A. strigosa* showed a single point mutation leading to the G134A or D25 N substitutions in VIN-1.1, respectively (Table 2). Moreover, translation of allele *Vin-C*^{c}*1a* (GenBank JQ679294) in *A. clauda* led to a polypeptide with the highest similarity (96.4 %) to VIN-1.2 in cv. Donata (Online Resource 5).

When amplified with primers positioned at the untranslated regions (UTRs) of gene *3B3-7* (PP4, Table 1), single plants of cvs Donata and Primula gave the expected 528-bp products, which were cloned in *E. coli* and sequenced. Three *Vin-2* genes, i.e., *Vin-A2a* (GenBank JQ518370), *Vin-C2a* (GenBank JQ518371) and *Vin-D2a* (GenBank JQ518369) coding for VIN-2.1, VIN-2.2 and VIN-2.3, were found in each cultivar. These VIN-2 proteins, 147 residues in size, differ from each other and from proteins 3B3-5 and 3B3-7 by only 1–3 amino acid substitutions (Table 2). Single plants of diploid oat species amplified with the primer pair specific to gene *3B3-7* produced 528-bp-long amplicons as well. These amplicons revealed *Vin-A*^{h}*2a* (GenBank JQ679303) in *A. hirtula* and *Vin-A*^{s}*2a* (GenBank JQ679304) in *A. strigosa*, each allele coding for a polypeptide with 99.3–100 % similarity with VIN-2.1 in *A. sativa* (Online Resource 5). Furthermore, alleles *Vin-A*^{i}*2a* (GenBank JQ679298) and *Vin-C*^{i}*2a* (GenBank JQ679299) in *A. insularis* were shown to code for VIN-2.1 and VIN-2.2, respectively. These proteins are 99.3–100 % similar to their counterparts in cvs Donata and Primula. Finally, C-genome species *A. eriantha* exhibited single allele *Vin-C*^{e}*2a* (GenBank JQ679302), which codes for a polypeptide with only two amino acid substitutions with respect to VIN-2.2 in *A. sativa.*

PCR amplification of genomic DNA from single plants of cvs Donata and Primula with the primer pair positioned at the ends of the CDS of gene *3B3T-3* (PP5, Table 1) produced 426-bp-long amplicons, which were cloned in *E. coli* and sequenced. Two *Vin-3* genes were found in each cultivar. In particular, cvs Donata and Primula shared allele *Vin-A3a* (GenBank JQ518372), which codes for protein VIN-3.1. This protein is identical to the VIN-3.1 polypeptide encoded by allele *Vin-A*^{c}*3a* (GenBank KF032537) in A-genome *A. canariensis,* except for a single F9A substitution in the signal peptide (Table 2). Moreover, two different alleles, *i.e. Vin-C3a* (GenBank JQ518374) in cv. Primula and *Vin-C3b* (GenBank JQ518373) in cv. Donata were shown to code for two VIN-3.2 isoforms, 95.8 % similar

Table 3 Amino acid sequences of avenoindolines in *A. sativa* and other oat species

Protein	Gene	Species	Amino acid sequence							
Avenoindoline-A										
AIN-A1	*Aina-A1a*	*A. sativa*	MKALFLIGLL^{10}	ALVASTAFAQ^{20}	YSEVVGSYDV^{30}	AGGGGAQQCP^{40}	VETKLNSCRN^{50}	YLLDRCSTMK^{60}	DFPVTWRWWK^{70}	WWKGGC-QELL^{80}
	Aina-A^{h}1a	*A. hirtula*	- - - - - - - - - -	- - - - - - - - - -	- - - - - G - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -
	Aina-A^{s}1a	*A. strigosa*	- - - - - - - - - -	- - - - - - - - - -	- - - - - G - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -
	Aina-A^{i}1a	*A. insularis*	- - - - - - - - - -	- - - - - - - - - -	- - - - - G - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -
AIN-A2	*Aina-C1a*	*A. sativa*	- - - - - - - - - -	- - - - - - - - - -	- - - - - D - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -
AIN-A1	*Aina-A1a*	*A. sativa*	GECCSRLGQM^{90}	PPQCRCNIIQ^{100}	GSIQGDLGGI^{110}	FGFQRDRASK^{120}	VIQEAKNLPP^{130}	RCNQGPPCNI^{140}	PGTIGYYW^{148}	
	Aina-A^{h}1a	*A. hirtula*	- - - - - - L - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - -	
	Aina-A^{s}1a	*A. strigosa*	- - - - - - L - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - -	
	Aina-A^{i}1a	*A. insularis*	- - - - - - L - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - -	
AIN-A2	*Aina-C1a*	*A. sativa*	- - - - - - F - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - - - -	- - - - - - - -	
Avenoindoline-B										
AIN-B1	*Ainb-A1a*	*A. sativa*	MKTLFLLALL^{10}	ALVASTTFAQ^{20}	YSEVGGWYNE^{30}	VGAGGSSQQC^{40}	PLGRPKLSSC^{50}	KDYVMGWCFT^{60}	MKDFPFTWPT^{70}	KWWKG-GCEHE^{80}
	Ainb-A^{s}1a	*A. strigosa*	- - - - - - - - - -	- - - - S - - - - -	- - - - - - - - - -	- - - - - S - - - -	-LG - - K - - - -	- - - - - GW - - -	- - - - - F - - - -	K - - - - - - - - -
	Ainb-A^{i}1a	*A. insularis*	- - - - - - - - - -	- - - - S - - - - -	- - - - - - - - - -	- - - - - S - - - -	-LE - - K - - - -	- - - - - GW - - -	- - - - -F - - - -	K - - - - - - - - -
AIN-B2	*Ainb-C1a*	*A. sativa*	- - - - - - - - - -	- - - - G - - - - -	- - - - - - - - - -	- - - - - G - - - -	-ME - - N - - - -	- - - - - ER - - -	- - - - -V - - - -	R - - - - - - - - -
	Ainb-C^{i}1a	*A. insularis*	- - - - - - - - - -	- - - - G - - - - -	- - - - - - - - - -	- - - - - G - - - -	-ME - - N - - - -	- - - - - ER - - -	- - - - -V- - - -	R - - - - - - - - -
AIN-B3	*Ainb-D1a*	*A. sativa*	- - - - - - - - - -	- - - - S - - - - -	- - - - - - - - - -	- - - - - S - - - -	-LE - - K - - - -	- - - - - GW - - -	- - - - -F - - - -	K - - - - - - - - -
AIN-B1	*Ainb-A1a*	*A. sativa*	VRENCCKQLS^{90}	QIAPQCRCDS^{100}	IRGMIQGKLG^{110}	GFFGIWRGDV^{120}	FKKIQRAQSL^{130}	PSKCNMGADC^{140}	KFPSGYYW^{148}	
	Ainb-A^{s}1a	*A. strigosa*	- - - N - -K - - -	- - - - - - - C - -	- - - - - - - - - -	- - - - - - - - - -	- - K- - - - - S-	- - - - - - - - - -	- - - - - - - -	
	Ainb-A^{i}1a	*A. insularis*	- - - N- - K- - -	- - - - - - - C - -	- - - - - - - - - -	- - - - - - - - - -	- - K- - - - - S-	- - - - - - - - - -	- - - - - - - -	
AIN-B2	*Ainb-C1a*	*A. sativa*	- - - K - -Q - - -	- - - - - - - C - -	- - - - - - - - - -	- - - - - - - - - -	- - Q - - - - - R-	- - - - - - - - - -	- - - - - - - -	
	Ainb-C^{i}1a	*A. insularis*	- - - K - -Q - - -	- - - - - - - C - -	- - - - - - - - - -	- - - - - - - - - -	- - Q - - - - - R-	- - - - - - - - - -	- - - - - - - -	
AIN-B3	*Ainb-D1a*	*A. sativa*	- - - N- - K- - -	- - - - - - - S - -	- - - - - - - - - -	- - - - - - - - - -	- - K- - - - - S-	- - - - - - - - - -	- - - - - - - -	

Table 4 Mean SKCS values of T_1 kernels of transgenic durum wheat cv. Svevo

Transgenes [a]	VIN [b]		No. of T_0 plants	No. of T_1 kernels	Mean SKCS value ± SD	SKCS range	
	2.3	3.1				Min	Max
bar, Vin-D2a, Vin-A3a	+	+	16	160	66.8 ± 19.5**	−2.2	112.3
bar, Vin-D2a, Vin-A3a	+	−	1	10	65.3 ± 7.6	55.2	84.4
bar, Vin-D2a, Vin-A3a	−	+	1	10	69.1 ± 15.5	43.4	92.3
bar, Vin-A3a	−	+	3	30	76.4 ± 14.1 ns	47.0	109.1
bar	−	−	9	90	87.7 ± 12.3	49.2	115.1
Control cv. Svevo	−	−	5	300	91.0 ± 11.9 ns	48.5	110.5

** t value significant at $P < 0.01$ with respect to transgenics with *bar* only; ns, t value not significant with respect to transgenics with *bar* only

[a] Transgenes detected in T_0 plants by PCR

[b] Vromindolines detected in T_1 kernels by immunoblotting (+ = present, − = absent)

to each other. Finally, allele *Vin-C*^{i}*3a* (GenBank JQ679305) coding for a protein identical to VIN-3.2 in cv. Primula, was discovered in *A. insularis*. In this species, allele *Vin-A*^{i}*3a* (GenBank JQ679306) codes for a VIN-3.1 protein identical to 3B3T.

Vin genes in other cereal species

Primer pairs PP3, PP4 and PP5 (Table 1) specific for the *Vin-1*, *Vin-2* and *Vin-3* genes, respectively, were used to amplify genomic DNA of *Aegilops* species (*Ae. columnaris*, *Ae. speltoides*, *Ae. tauschii*, *Ae. trivialis*, *Ae. truncialis* and *Ae. ventricosa*), *Hordeum vulgare*, *Oryza sativa*, *Secale cereale, Sorgum vulgare* and *Triticum* species (*T. monococcum, T. timopheevii, T. turgidum* ssp *durum*, *T. urartu* and *T. zhucovskyi*). No amplicons were detected among the 15 cereal species analyzed except that accessions ID508, ID331 and Monlis of A^m-genome *T. monococcum* produced a PCR fragment of 447 base pairs when amplified with primer pair PP4 specific to gene *Vin-2* (data not shown). Traces of this fragment were found in hexaploid A^mA^uG-genome *T. zhucovskyi* as well. DNA sequencing of the amplicons from 'monococcum' wheat revealed a gene identical to *Vin-D2a*. However, RT-PCR amplification of 14-DPA-old kernels of accession ID331 produced minimal amounts of *Vin-2* transcripts. In addition, the A-PAGE fractionation of starch-bound proteins from accession ID331 gave no reaction with the anti-3B3 antibody specific to VIN-2.

Vromindoline and kernel texture

Particle bombardment of 1,863 embryo-derived calli of durum wheat cv. Svevo produced 44 T_0 independent transgenic plants resistant to bialaphos, with a transformation efficiency of 2.36 %.

Thirty of these plants were screened for the presence of *Vin-D2a* and *Vin-A3a* by PCR, and found to consist of 18 individuals with all three transgenes, three individuals with *bar* and *Vin-A3a*, and nine individuals with *bar* only (Table 4). PCR screening of the 57 progeny of two T_0 plants (MJ-1 and MJ-2) with *bar, Vin-D2a* and *Vin-A3a* revealed these three transgenes in 40 progeny seedlings (21 progeny from MJ-1 plus 19 from MJ-2) and their absence in 17 seedlings (7 progeny from MJ-1 plus 10 from MJ-2). These ratios agree well with the expected 3:1 ratio if the introduced transgenes reside at a single locus. A-PAGE fractionations of proteins bound to the starch granules extracted from 10 crushed T_1 kernels of each T_0 plant were tested for the presence of VIN-2 and VIN-3 by immune reactions with their specific antisera. In addition, evidence of segregation has been obtained by comparing 10 single kernels from each T_0 plant for their hardness as determined by SKCS (Table 4). Transgenics immune-positive for VIN-2/VIN-3 tested significantly softer (mean SKCS = 66.8, t value = 3.64, $P < 0.01$) than those lacking vromindolines (mean SKCS = 87.7). For these hardness measurements, the SKCS values of progeny positive for VIN-2/VIN-3 ranged from −2.2 to 112.3, standard deviation being as high as 19.5. By contrast, mean, range and standard deviation of the SKCS values in the T_1 progeny without vromindolines were comparable to those in control cv. Svevo. Finally, two T_0 plants with all three transgenes produced T_1 seeds expressing either VIN-2.3 or VIN-3.1, and exhibited mean SKCS values (50 single kernels) (65.3 and 69.1, respectively) similar to that of kernels positive for both VIN proteins. On the other hand, three T_0 individuals with transgenes *bar* and *Vin-A3a* produced VIN-3.1 positive kernels with a texture slightly softer, but not statistically different, with respect to T_1 grains lacking vromindolines (Table 4).

When grown under chamber conditions, the 34 T_1 progeny of 2 transgenic T_0 plants with expression of both VIN-2.3 and VIN-3.1 were indistinguishable from control cv. Svevo for their morpho-physiological traits, fertility and seed set. Weight and test weight of T_2 kernels were comparable to those of cv. Svevo as well (Table 5). By contrast, nine plants

Table 5 Mean values (± SD) of weight, test weight and SKCS index of kernels produced by 34 T_1 plants from two T_0 plants of durum wheat cv. Svevo expressing *Vin-D2a* and *Vin-A3a*

Genotype of T_1 plants[a]	No. of T_1 plants	T_2 kernels			SKCS	SKCS range	
		Mean weight (mg)	Test weight (Kg/HL)	No. of kernels/spike		Min	Max
Homozygous	9	45.1 ± 5.3 ns	79.3 ± 0.9 ns	38.5 ± 6.9 ns	37.1 ± 12.5**	−11.3	72.2
Heterozygous	20	47.0 ± 5.9	82.0 ± 1.9	42.1 ± 7.4	58.3 ± 23.4	0.4	115.7
Null	5	44.8 ± 6.0	79.5 ± 1.2	40.2 ± 5.0	82.2 ± 12.9	49.3	117.6
Cv. Svevo[b]	5	45.2 ± 5.5 ns	79.8 ± 0.7 ns	43.7 ± 8.8 ns	88.8 ± 15.2 ns	41.1	110.2

** *t* value significant at $P < 0.01$ with respect to the null T_2 progeny; ns, *t* value not significant with respect to the null T_2 progeny

[a] For alleles *Vin-D2a* and *Vin-A3a*, as determined by PCR amplification of T_2 plantlets

[b] Non-transgenic control

homozygous for *Vin-D2a* and *Vin-A3a* produced T_2 kernels significantly softer (mean SKCS = 37.1 ± 12.5, valuated on 100 single kernels) than 5 null sister plants (mean SKCS = 82.2 ± 12.9). As expected, seeds produced by 20 heterozygous T_1 plants exhibited contrasting hardness characteristics, with SKCS values ranging from 0.4 to 115.7. The colorimetric assay with cloro-naphthol revealed that the polyclonal antiserum specific to VIN-3 (anti-3B3T) reacts with the starch-bound proteins extracted from T_2 kernels homozygous for *Vin-D2a* and *Vin-A3a* and fractionated by A-PAGE (Online Resource 6, lanes 1 and 2). A weak reaction was observed with kernels produced by T_1 heterozygous plants (lanes 4 and 5), whereas no reaction was observed with T_2 kernels from null plants (lane 3) and durum wheat cv. Svevo (lane 7). The same results were obtained with the anti-VIN-2 (anti-3B3) antibody (data not shown).

Discussion

Increased grain softness in cereal crops was found to reduce milling costs and particle size following milling, and enhance flour yield and digestibility (Knowlton et al. 1996; Wondra et al. 1995). Oat kernels exhibit an extra-soft texture and among the 17 oat cultivars analyzed in this study, hardness index ranged from SKCS value as low as −36.9 to −14.0 (average value −22.7 ± 5.5), with the lowest SKCS being surveyed in cv. Harpoon. This extra-soft texture is largely responsible for groat breakage during mechanical harvesting, threshing and dehulling, and negatively affects milling yield (proportion of unbroken naked kernels for a given amount of raw material), as well as size and quality of oat flakes. In addition, wounded caryopses show reduced germination and viability, and attract fungus invasion, raising the risk of mycotoxins in food and feed. Therefore, taking into account the increasing interest of oats as a healthy food, elucidation of the molecular bases of kernel texture in this cereal crop is of paramount importance from a technological point of view.

Grain texture in common and monococcum wheat is largely modulated by expression of *Pina-D1* and *Pinb-D1* genes and by accumulation in the endosperm of their encoded puroindolines, with five or three tryptophan residues in the TRD. Genes homologous to *Pina-D1* and *Pinb-D1*, i.e., *Aina* and *Ainb* (Gautier et al. 2000), also occur in oat species at diploid, tetraploid or hexaploid level. Here, using DNA extracted from single plants, the 6× polyploid *A. sativa* was found to host three copies of the *Ainb-1* gene likely mapping to three homoeologous chromosomes of the A, C and D genomes. In particular, genes *Ainb-A1a* (JX096494) in *A. sativa* and *Ainb-A^{s}1a* (JX 096496) in *A. strigosa* code for the same AIN-B1 polypeptide, which is 99.3 % similar to the AIN-B1 variant encoded by gene *Ainb-A^{i}1a* (JX096497) in *A. insularis* (Online Resource 5), suggesting A-genome location of these genes. The present AIN-B1 variants show 14–15 amino acid substitutions (89.9–90.5 % similarity) with respect to PIN-B encoded by allele *Pinb-D1a* in *T. aestivum*, but exhibit high similarity (99.3–100 %) to the PIN-B variant encoded by *Pinb-D1i* in *Aegilops tauschii* (Massa et al. 2004), the diploid progenitor of the D genome of common wheat.

In addition, translation of genes *Ainb-C1a* (JX 096495) in *A. sativa* and *Ainb-C^{i}1a* (JX096498) in *A. insularis* led to the same AIN-B2 protein, suggesting C-genome location of this pair of genes. Notably, *Ainb-C1a* exhibits the same CDS as the *Pinb-N1a* gene placed on chromosome 5 N of tetraploid DN-genome species *Aegilops ventricosa* (Gazza et al. 2006). This raises the possibility that C-genome species *A. ventricosa*, *A. clauda* and *A. eriantha* may have a certain phylogenetic relationship with N-genome *Aegilops uniaristata*. The AIN-B2 sequence showed reduced similarity to AIN-B1 (91.2 %) and wild-type PIN-B (92.6 %).

The donor of A-genome euchromatic chromosomes of *A. sativa* has been identified in diploid species *A. strigosa* or *A. canariensis*, whereas the diploid species *A. ventricosa* and *A. eriantha* have been proposed as the putative donors of the heterochromatic C-genome chromosomes (Rajhathy and Thomas 1974; Thomas 1992; Nikoloudakis and

Katsiotis 2008). The absence of D-genome diploid species and the high homology between the A and D genomes (Jellen et al. 1994) led to the viewpoint that these two genomes of *A. sativa* have been inherited from an A-genome diploid species (Linares et al. 1998).

This is in accordance with the observation that the hexaploid oat was found to possess gene *Ainb-D1a* (JX096493) likely located to the D genome. This gene codes for an AIN-B3 sequence with only 2 amino acid substitutions (98.6 % similarity) with respect to AIN-B1 encoded by the A genome. Translation of *Aina-A1a* (JX096490) in *A. sativa, Aina-A^{h}1a* (JX096499) in *A. hirtula*, *Aina-A^{s}1a* (JX096489) in *A. strigosa* and *Aina-A1^{i}a* (JX096492) in *A. insularis* led to the same AIN-A1 sequence, indicating that these genes are probably from the A genome. It is also noteworthy that AIN-A1 is identical to PIN-A encoded by allele *Pina-D1a* in common wheat, suggesting low variability within the A-type indolines. This is confirmed by the minor sequence polymorphism of AIN-A2 encoded by the *Aina-C1a* allele (JX096491), which is provisionally assigned to the C genome. Assuming the conjecture of a single *Aina* gene per genome and the substantial genetic similarity between the A and D genomes (Jellen et al. 1994), allele *Aina-D1a* encoding for an AIN-A3 polypeptide identical to AIN-A1 can be anticipated to occur in the D genome of hexaploid oats.

PIN-A and PIN-B directly bind to the starch granule surface, and this cellular localization would prevent adhesion between starch granules and the surrounding protein matrix, reducing firmness of wheat grain (Giroux and Morris 1998; Bhave and Morris 2008). Interestingly, no *Ainb* transcripts were detected in immature kernels of oat cvs Donata and Primula. Further, 2-D gel electrophoresis and mass spectrometry did not provide any evidence of AIN-B proteins on the starch granules in those cultivars. On the other hand, RT-PCR amplification and Western blotting revealed that oat kernels accumulate reduced amounts of *Aina* transcripts and AIN-A polypeptides, suggesting that avenoindolines A and B exert a negligible role, if any, in determining grain texture in hexaploid oats, in contrast with the function recognized to their homologous puroindolines in wheat.

Conversely, the present study shows that during the grain-filling stage diploid, tetraploid and hexaploid oat species accumulate the novel vromindolines proteins, with four tryptophan residues in the TRD, in the endosperm. These proteins of decreasing molecular weights are synthesized as precursors that undergo specific cleavage of the leader peptide and, probably, processing at both C- and N-terminal ends. In the A- and C-genome species analyzed here, each VIN is encoded by a single gene. Therefore, there should exist six *Vin* genes in AC-genome tetraploid oat species, and nine *Vin* genes in hexaploid ACD-genome oats. PCR amplification of genomic DNA from tetraploid *A. insularis* and hexaploid *A. sativa* supported this conclusion, except that oat cvs Primula and Donata revealed only two different gene sequences at the *Vin-3* locus. However, this could be accounted for by the very high similarity between A and D genomes.

VIN-1.1 (allele *Vin-A1a*) and VIN-2.1 (allele *Vin-A2a*) are likely encoded by the A genome of *A. sativa*, as suggested by their high similarity to vromindolines in *A. hirtula* and *A. strigosa*. These VIN polypeptides were found to have the highest similarity to VIN-1.3 and VIN-2.3 encoded by *Vin-D1a* and *Vin-D2a* on the D genome, respectively. By contrast, the strong genetic divergence of the C genome from the A/D genome (Jellen et al. 1994; Nikoloudakis and Katsiotis 2008) was paralleled by a high number of polymorphisms identified in the amino acid sequence of VIN-1.2 and VIN-2.2 with respect to their counterparts mentioned above.

The presence of vromindolines genes in all the three oat genomes and the accumulation of large amounts of VIN-1, VIN-2 and VIN-3 polypeptides suggests a primary role of these polypeptides in the determination of the extra-soft texture of oat groats. A preliminary assessment of proteins bound to 12 mg of starch granules as determined by the densitometric analysis of A-PAGE fractionations, revealed that the amount of vromindolines in oats cvs Donata and Primula is roughly threefold higher than that of puroindolines (PIN-A plus PIN-B) in soft-textured wheat cvs Bolero and Bilancia (data not shown). Further, expression of transgenes *Vin-D2a* and *Vin-A3a* and accumulation of their encoded proteins VIN-2.3 and VIN-3.1 on the starch granules caused a strong reduction (about 50 %) of grain hardness in the extra-hard durum wheat cv. Svevo, which does not contain any *Pina* or *Pinb* homologs. The seed phenotype of transgenic durum wheat was stable and heritable, suggesting that vromindolines can be used to modify the texture of other cereals such as rice, corn, sorghum and common wheat.

Moreover, it is notable that oat endosperm differs from that of other cereals in possessing a single population of starch granules of irregular shape, often polyhedral but also ovoid or hemispherical. The individual starch granules, 3–10 µm in size, can form aggregates of 60 µm in diameter (Zhou et al. 1998). In addition, oat endosperm is characterized by a high amount of polar lipids associated with starch compared with wheat (Gudmundsson and Eliasson 1989). In common wheat, polar lipids on the surface of water-washed starch are able to interact with the TRD of puroindolines, and thus modulate the soft/hard endosperm texture. In particular, starch from soft wheats was found to have high amounts of polar lipids compared with hard wheats (Greenblatt et al. 1995). The high amount of both polar lipids and vromindolines could synergically contribute in determining the extra-soft texture of oat grains. In addition as compared with the ovoid granules typical of

wheat, barley and rye those of oats have a polyhedral shape which would increase the surface/volume ratio. Finally, VIN proteins reveal significant heterogeneities, which could affect their lipid-binding properties. In this contest, it is noteworthy that single residue substitutions in or near to the TRD were found to affect interaction of PIN-A and PIN-B with yeast plasma membranes or phospholipids monolayers (Clifton et al. 2007; Evrard et al. 2008).

Vin gene, allele *Vin-D2a* coding for VIN-2.3, was found in *Triticum monococcum* ssp *monococcum* (A^m genome), indicating that genes coding for vromindolines are not unique to the genus *Avena*. A weak *Vin-2* amplicon was obtained in *T. zhucovskyi* (A^mA^uG genome) as well. This is suggestive of a phylogenetic relationship between the A^m genome of monococcum wheat and the A/D genome of *Avena* species. However, no *Vin-D2a* transcripts were found in the developing seeds of monococcum wheat. Therefore, *T. monococcum* and *A. sativa* share indoline genes *Pina*, *Pinb,* and *Vin* but follow contrasting strategies in expression of these genes, and preferentially accumulate either puroindolines (monococcum wheat) or vromindolines (oats) on their starch granules.

It could be summarized that the *Hordeae* and *Aveneae* tribes are unique in showing a multiform indoline protein family, named tryptophanins by Tanchak et al. (1998), which consists of endosperm proteins sharing a highly conserved skeleton of 10 cysteine residues as well as a hydrophobic TRD containing 2–5 tryptophan residues. Among these tryptophan-rich proteins, PIN-B2 proteins (Wilkinson et al. 2008) and GSPs from wheat have two W residues in their TRD, whereas AIN-B, HIN-B1/HIN-B2 and PIN-B from oats, barley and wheat, respectively, show three W residues. On the other hand, VIN proteins from oats are unique in having four W residues in their TRD, whereas AIN-A, HIN-A and PIN-A from oats, barley and wheat, respectively, possess five W residues. Only indoline proteins with a number of W residues ≥ 3 in their TRD seems to be able to interact with structural lipids of amyloplast, and affect kernel texture (Wilkinson et al. 2008; Giroux and Morris 1998). In oats, this role seems to be played by vromindolines.

In conclusion, the present work offers an overall framework of oat indolines in the genus *Avena* and of their effect on grain texture, a trait of great trade importance in cereal crops. The results demonstrate that the extra-soft oat kernel texture is largely modulated by the novel vromindoline proteins, characterized by a TRD with four W residues. In addition, evidence of a suggestive phylogenetic relationship between members of *Hordeae* and *Aveneae* tribes has been obtained.

The vromindolines genes identified in this work can be used to genetically modify the kernel texture of other cereals such as barley, wheat, rice, corn and sorghum, as demonstrated here by the softening effect induced by two vromindoline genes in durum wheat. Finally, the molecular characterization of vromindoline genes in oat species at different ploidy levels will contribute to better understand the evolution of the *Avena* genome.

References

Anderson DD, Greene FC, Yip RE, Halford NG, Shewry PR, Malpica-Romero JM (1989) Nucleotides sequences of the two high-molecular-weight glutenin genes from the D-genome of a hexaploid bread wheat, *Triticum aestivum* L. cv Cheyenne. Nucleic Acids Res 17:461–462

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1994) Genomic extraction using the modified CTAB method. Current protocols in molecular biology. Wiley, New York

Bhave M, Morris CF (2008) Molecular genetics of puroindolines and related genes: regulation of expression, membrane binding properties and applications. Plant Mol Biol 66:221–231

Blochet JE, Chevalier C, Forest E, Pebay-Peyroula E, Gautier MF, Joudrier P, Pezolet M, Marion D (1993) Complete amino acid sequence of puroindoline, a new basic and cysteine-rich protein with a unique tryptophan-rich domain, isolated from wheat endosperm by Triton X-114 phase partitioning. FEBS Lett 329:336–340

Chantret N, Salse J, Sabot F, Rahman S, Beliec A, Laubin B, Dubois I, Dossat C, Sourdille P, Joudrier P, Gautier MF (2005) Molecular basis of evolutionary events that shaped the hardness locus in diploid and polyploid wheat species (*Triticum* and *Aegilops*). Plant Cell 17:1033–1045

Chen F, He ZH, Xia XC, Lillemo M, Morris CF (2005) A new puroindoline b mutation present in Chinese winter wheat cultivar Jingdong 11. J Cereal Sci 42:267–269

Clifton LA, Lad MD, Green RJ, Frazier RA (2007) Single amino acid substitutions in puroindoline-b mutants influence lipid binding properties. Biochem 46:2260–2266

Corona V, Gazza L, Boggini G, Pogna NE (2001) Variation in friabilin composition as determined by A-PAGE fractionation and PCR amplification, and its relationship to grain hardness in bread wheat. J Cereal Sci 34:243–250

Dexter JE, Preston KR, Martin DG, Gander EJ (1994) The effects of protein content, and starch damage on the physical dough properties and breadmaking quality of Canadian durum wheat. J Cereal Sci 20:139–151

Egorov TA, Odintsova TI, Musolyamov AKh, Fido R, Tatham AS, Shewry PR (1996) disulphide structure of a sunflower seed albumin: conserved and variant disulphide bonds in the cereal prolamin superfamily. FEBS Lett 396:285–288

Evrard A, Lagarde V, Joudrier P, Gautier M-F (2008) Puroindoline-a and puroindoline-b interact with *Saccharomyces cerevisiae* plasma membrane through different amino acids present in their tryptophan-rich domain. J Cereal Sci 48:379–386

Gautier MF, Cosson P, Guirao A, Alary R, Joudrier P (2000) Puroindoline genes are highly conserved in diploid ancestor wheats and related species but absent in tetraploid *Triticum* species. Plant Sci 153:81–91

Gazza L, Nocente F, Ng PKW, Pogna NE (2005) Genetic and biochemical analysis of common wheat cultivars lacking puroindoline a. Theor Appl Genet 110:470–478

Gazza L, Conti S, Taddei F, Pogna NE (2006) Molecular characterization of puroindolines and their encoding genes in *Aegilops ventricosa*. Mol Breed 17:191–200

Giroux MJ, Morris CF (1998) Wheat hardness results from highly conserved mutations in the friabilin components puroindolines a and b. Proc Nat Acad Sci USA 95:6262–6266

Greenblatt GA, Bettge AD, Morris CF (1995) The relationship among endosperm texture, friabilin occurrence, and bound polar lipids on wheat starch. Cereal Chem 72:172–176

Gudmundsson M, Eliasson AC (1989) Some physico-chemical properties of oat starches extracted from varieties with different oil content. Acta Agric Scand 39:101–111

Hansen HB, Møller B, Andersen SB, Jørgensen JR, Hansen A (2004) Grain characteristic, chemical composition, and functional properties of rye (*Secale cereale* L.) as influenced by genotype and harvest year. J Agric Food Chem 52:2282–2291

Iwami A, Osborne BG, Huynh H-N, Andersen RS, Wesley IJ, Kajiwara Y, Takashita H, Omori T (2005) The measurement of structural characteristics of barley for Shochu using Single-kernel characterization system 4100 crush-response profiles. J Inst Brew 111:181–189

Janni M, Sella L, Favaron F, Bleachl AE, De Lorenzo G, D'Ovidio R (2008) The expression of a bean pgip in transgenic wheat confers increased resistance to the fungal pathogen *Bipolaris sorokiniana*. MPMI 21:171–177

Jellen EN, Gill BS, Cox TS (1994) Genomic in situ hybridization differentiates between A/D- and C-genome chromatin and detects intergenomic translocation in polyploid oat species (genus *Avena*). Genome 37:613–618

Knowlton KF, Allen MS, Erickson PS (1996) Lasalocid and particle size of corn grain for dairy cows in early lactation.1. Effect on performance, serum metabolite, and nutrient digestibility. J Dairy Sci 79:557–564

Ladizinsky G (1998) A new species of *Avena* from Sicily, possible the tetraploid progenitor of hexaploid oats. Genet Res Crop Evol 45:263–269

Li PX-P, Hardacre AK, Campanella OH, Kirkpatrick KJ (1996) Determination of endosperm characteristics of 38 corn hybrids using the stenvert hardness test. Cereal Chem 73:466–471

Lillemo M, Simeone MC, Morris CF (2002) Analysis of puroindoline a and b sequences from *Triticum aestivum* cv. 'Penawawa' and related diploid taxa. Euphytica 126:321–331

Linares C, Ferrer E, Fominaya A (1998) Discrimination of the closely related A and D genomes of the hexaploid oat *Avena sativa* L. Proc Nat Acad Sci USA 95:12450–12455

Lindahl L, Eliasson A-C (1992) A comparison of some rheological properties of durum and wheat flour doughs. Cereal Chem 69:30–34

Massa AN, Morris CF (2006) Molecular evolution of the puroindoline-a, puroindoline-b, and grain softness protein-1 genes in the tribe Triticeae. J Mol Evol 63:526–536

Massa AN, Morris CF, Gill BS (2004) Sequence diversity of Puroindoline-a, Puroindoline-b, and the grain softness protein Genes in *Aegilops tauschii* coss. Crop Sci 44:1808–1816

Mohammadi M, Abbas Zaidi M, Ochalski A, Thanchak M, Altosaar I (2007) Immunodetection and immunolocalization of tryptophanins in oat (*Avena sativa* L.) seeds. Plant Sci 172:579–587

Nikoloudakis N, Katsiotis A (2008) The origin of the C genome and cytoplasm of *Avena* polyploids. Theor Appl Genet 117:273–281

Pedersen JF, Martin CR, Felker FC, Steele JL (1996) Application of the single kernel wheat characterization technology to sorghum grain. Cereal Chem 73:421–423

Peltonen-Sainio P, Muurinen S, Vilppu M, Rajala A, Gates F, Kirkkari AM (2001) Germination and grain vigour of naked oat in response to grain moisture at harvest. J Agric Sci 137:147–156

Peterson DM (1992) Composition and nutritional characteristics of oat grain and products. Oat science and Technology. Marshall HG and Sorrels ME (Co-Eds) Madison, USA, pp 265–292

Peterson DM (2004) Oat—a multifunctional grain. In: Proceedings 7th Int Oat Conference. Pelton-Sainio P and Topi-Hulmi M (Eds). MTT Agrifood Research Finland, pp 21–26

Pogna NE, Gazza L, Corona V, Zanier R, Niglio A, Mei E, Palumbo M, Boggini G (2002) Puroindolines and kernel hardness in wheat species. In: Ng PKW, Wrigley CW (eds) Wheat quality elucidation: the Bushuk legacy. AACC, Inc., St. Paul, pp 155–169

Rahman S, Jolly GJ, Skerritt JH, Waelosheck A (1994) Cloning of a wheat 15-KDa grain softness protein (GSP). J Biochem 223:917–925

Rajhathy T, Thomas H (1974) Cytogenetics of oats (*Avena* L.). Misc Publ Genet Soc Can 2:1–90

Redaelli R, Sgrulletta D, Scalfati G, De Stefanis E, Conciatori A, Cammerata A, Pollini CM (2005) L'avena per un prodotto alimentare ricco di proprietà salutari. Risultati della ricerca in Italia. Tecnica Molitoria 3:257–267 (In Italian)

Rondanelli M, Opizzi A, Monteferraio F (2009) The biological activity of beta-glucans. Minerva Med 100:237–245

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Annal Chem 68:850–858

Taddei F, Gazza L, Conti S, Muccilli V, Foti S, Pogna NE (2009) Starch-bound 2S proteins and kernel texture in einkorn, *Triticum monococcum* ssp *monococcum*. Theor Appl Genet 119:1205–1212

Tanchak MA, Schernthaner JP, Giband M, Altosaar I (1998) Tryptophanins: isolation and molecular characterization of oat cDNA clones encoding proteins structurally related to puroindoline and wheat grain softness proteins. Plant Sci 137:173–184

Thomas H (1992) Cytogenetics of *Avena*. In: Oat science and technology. Marshall HG and Sorrells ME (Eds). Agron Monogr 33. ASA and CSSA, Madison, WI, pp 473-507

Tosi P, D'Ovidio R, Napier JA, Bekes F, Shewry PR (2004) Expression of epitope tagged LMW glutenin subunits in the starchy endosperm of transgenic wheat and their incorporation into glutenin polymers. Theor Appl Genet 108:468–476

Valentine J (1995) Naked oats. In: Welch RW (ed) The oat crop: production and utilization. Springer, Netherlands, pp 504–532

Valentine J, Hale OD (1990) Investigations into reduced germination of seed of naked oats. Plant Var Seed 3:21–30

Wilkinson M, Wan Y, Tosi P, Leverington M, Snape J, Mitchell RAC, Shewry PR (2008) Identification and genetic mapping of variant forms of puroindoline b expressed in developing wheat grain. J Cereal Sci 48:722–728

Wondra KJ, Hancock JD, Behnke KC, Hines RH, Stark CR (1995) Effects of particle size and pelleting on growth performance, nutrient digestibility, and stomach morphology in finishing pigs. J Anim Sci 73:757–763

Zhou M, Robards K, Glennie-Holmes M, Helliwell S (1998) Structure and pasting properties of oat starch. Cereal Chem 75:273–281