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GM Crops: From basic research to application

Manipulating Rab GTPase activity in wheat to improve gluten quality for breadmaking

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Summary

Sequence information of Rab GTPases in *Arabidopsis*, rice, *Brachypodium* and bread wheat was compiled by various means. These included online BLAST and string searches for genes, proteins, ESTs (expressed sequence tags), unigenes and GSSs (genome survey sequences). Sequences of 56 *Rab* genes from *Arabidopsis*, 44 from rice, 41 from *Brachypodium*, three from wheat and 26 wheat unigenes were collected. The sequence information was used to produce dendrograms from amino acid and nucleic acid sequences for comparison of *Rab* subfamilies in these species.

A suitable candidate gene (*Ta.54382*) was chosen as the target for down-regulation. An RNAi construct was produced, targeting a region of the gene, and used to transform wheat explants.

Key words: Rab GTPase, *Triticum aestivum*, seed storage protein, protein transport, gluten

Introduction

Functions of the Rab GTPase family

In the Ras superfamily of small GTPases, plants contain Rab, Rho, Arf, and Ran GTPases but not the Ras subfamily present in mammals (Vernoud *et al*., 2003; Yang, 2002). The plant Rab GTPases are best characterised in *Arabidopsis thaliana* with 56 members, each of which is considered to be involved in a particular trafficking step. Rab GTPases are molecular switches that regulate the transport of vesicles between membranous compartments of the exocytic and endocytic pathways of eukaryotes. As well as vesicle budding, tethering and docking (Zerial & McBride, 2001) Rab proteins are also involved in the regulation of vesicle and compartment motility through recruitment of motor proteins to the membrane (Stenmark, 2009).

There are eight clades of Rab GTPase in plants, designated A–H. They are generally related to mammalian Rab classes 11, 2, 18, 1, 8, 5, 7 and 6 respectively (Pereira-Leal & Seabra, 2001). Early plant secretory traffic involves GTPases of the Rab B and Rab D clades (Rutherford & Moore, 2002) which have been shown to target proteins to the Golgi (Cheung *et al*., 2002; Zheng *et al*., 2005).

The Rab D clade in plants is equivalent to Rab1 in animals and Ypt1 in yeasts, and is split into two subclades - Rab D1 and Rab D2. Rab D2a in plants is analogous to mammalian Rab1B and has been localised to the ER and Golgi. The presence of Rab1 proteins on both Golgi and ER compartments has been reported, which suggests a role in trafficking between the two compartments

(Plutner *et al*., 1991). In yeast, Ypt1p affects docking of ER-derived vesicles and also regulates Uso1p-dependent tethering of donor vesicles to the target membrane (Cao *et al*., 1998) suggesting the mediation of Ypt1p in tethering of ER-derived vesicles before membrane fusion occurs. Several studies have demonstrated a similar role for Rab1B - the mammalian ortholog of Ypt1p. It was shown to mediate the docking of ER-derived vesicles with the cis-Golgi compartment by recruitment of cytosolic tethering factors (Allan *et al*., 2000) and displayed interaction with Golgi membrane proteins (Moyer *et al*., 2001). In another study Rab1-specific antibodies blocked ER to the Golgi and also intra-Golgi trafficking (Plutner *et al*., 1991).

The role of *Arabidopsis* Rab D2a in traffic from the ER to the Golgi was demonstrated by the over-expression of a dominant-negative form of Rab D2a (N121I) which resulted in accumulation of secreted and Golgi markers in an ER-like reticulate compartment (Batoko *et al*., 2000). However, inhibition by the dominant-negative in several other species did not affect trafficking to the protein storage vacuole in leaf protoplasts (Park *et al*., 2004), which would indicate independent secretory traffic pathways. The trafficking of peroxisomal membrane proteins AtPEX2 and AtPEX10, suggested to be transported to the peroxisome via the ER, was shown insensitive to the dominant-negative Rab D2a as well as a drug causing the ER and Golgi to fuse (brefeldin A) (Sparkes *et al*., 2005).

In a study on the tetraploid durum wheat by Di Luccia *et al*. (2005) a dominant-negative *Rab1B* gene (orthologue of *Arabidopsis RabD2*) from tobacco was thought to influence the transport of gluten proteins within the secretory system by down-regulating the trafficking step from the ER to the Golgi. The result was an alteration in functional properties of the grain compared to the wild type control displaying improved gluten quality.

Seed storage proteins of cereals and wheat

During development the starchy endosperm of a wheat seed accumulates important energy and protein stores. Approximately 70% of the dry weight of cereal seed is starch while the protein content varies more depending on species and variety. Storage protein generally accounts for 10−15% of the dry mass of wheat seeds (Shewry & Halford, 2002).

The important gluten proteins of wheat are members of the prolamin superfamily (Kreis *et al*., 1985). They are divided into the monomeric gliadins, which only contain intra-chain disulphide bonds, and polymeric glutenins. Glutenin proteins form inter-chain disulphide bonds which enable a complex polymer structure. Glutenin subunits are divided into high molecular weight (HMW-GS) and low molecular weight (LMW-GS) subunits. Gliadins are sub-classified as α-, β-, γ- and ω-, depending on fractionation by acidic PAGE (Woychik *et al*., 1961). Gluten proteins are synthesised by membrane-bound polyribosomes on the RER before moving into the ER lumen. Within the ER processing occurs including folding, disulphide bond formation (including inter-chain bonds in glutenins) and hydrogen bonding. The latter can cause the gluten protein to precipitate, forming hydrated protein particles (Tosi *et al*., 2009).

Rubin *et al*. (1992) proposed that two different types of protein body appear to accumulate in developing wheat endosperm. The first contains relatively dense protein and seems to bud off directly from protein aggregates in the ER that, it is suggested, are too large and insoluble to enter the Golgi apparatus. HMW glutenin subunits are found almost exclusively in these protein bodies throughout development. The second type is less dense and accounts for proteins trafficked via the Golgi.

Protein sorting into different protein bodies was also shown by Tosi *et al.* (2009) using one antibody specific to a LMW glutenin subunit and another, less specific antibody that recognised other gluten proteins. The study also showed that gluten trafficking via the Golgi is favoured during early seed development but later on, during the intense grain filling phase, protein aggregation in the RER was much increased, which suggests greater frequency of ER-derived protein bodies.

Gluten and baking

The prolamins of wheat - gliadins and glutenins - are the major components of the gluten protein fraction, which gives wheat its unique bread making and general baking qualities. The physical

properties of gluten are commonly described as a viscoelastic, i.e. a combination of elasticity and extensibility. The elasticity of the dough is responsible for trapping the CO_2 released by yeast during baking, causing the dough to rise, and is therefore a critical property of a good bread wheat. A 'weak' (less elastic) bread flour will let CO_2 escape, while an excessively strong flour confers so much elasticity that expansion is reduced. Both lead to bread failing to rise properly and therefore produce poor quality bread.

Though it is likely all the various gluten proteins have some effect on the dough, the elasticity and extensibility can generally be assigned to glutenins and gliadins respectively (Shewry *et al*., 1995). Wheat of good bread making quality is classically characterised by its high protein content, but also by the types of proteins present. Of the gluten proteins, most important for bread making are the HMW glutenin subunits (HMW-GS) as the types and amounts present have the greatest effect on the functional properties of the dough (Payne, 1987). Their sequences include a large central region of repeats, as well as C- and N- termini that include cysteine residues involved in disulphide bonding. The resulting structure has small disulphide-bonding domains rich in α-helices at the ends and a large central section of β-reverse turns where intra- and inter-chain hydrogen bonding occurs (Shewry *et al*., 1995). These features along with their size help explain how HMW-GS contribute to the elastic properties of gluten.

A study by Di Luccia *et al*. (2005) investigated the effect of co-suppression (down-regulation of endogenous gene expression through over-expression of a homologous gene) of *RabD2a* in durum wheat (*Triticum durum*) using a *RabD2a* gene from tobacco (*Nicotiana tabacum*). Through rheological tests the authors found a significant increase in gluten quality in semolina made from the transgenic grain, yet the levels of gluten proteins were unaltered. If a similar effect could be obtained in bread wheat, it would not only help towards improving our knowledge of the major trafficking steps of important seed storage proteins, but could also represent a substantial advance in our understanding of bread making and potentially contribute to plant improvement.

Materials & Methods

Gathering sequence data

Arabidopsis thaliana Rab GTPase proteins and their associated genes and coding sequences (CDS) were found mainly by string searches. After the *Arabidopsis* Rab database had been produced, subsequent sequence searches were mostly by BLAST (Basic Local Alignment Search Tool) using sequences already gathered. Full wheat Rab proteins and genes were sought using online bioinformatic search engines such as NCBI BLAST, followed by searching for wheat ESTs showing similarity to the *Arabidopsis Rab* genes. The unigene linked to each EST in the search results was noted then the sequences of all the ESTs belonging to each unigene were aligned using Geneious 4.8 (Drummond *et al*., 2010) to produce consensus sequences, which were then analysed to find CDS with Rab-like features.

Phylogenetic analysis

Phylogenetic analyses were carried out using Rab protein and nucleotide sequence data from *Arabidopsis thaliana*, *Oryza sativa, Brachypodium distachyon, Triticum aestivum* and *Nicotiana tabacum*. Alignments and phylogenetic tree files were generated using MAFFT version six online, L-INS-i method (Katoh & Toh, 2010). All trees were neighbour-joining with 1000 bootstrap replicates, shown on branches as percentage confidence values.

Primers & DNA

Primers were obtained from Eurofins MWG Operon (Anzingerstr. 7a, 85560 Ebersberg, Germany). dNTPs used in PCR were obtained from Promega (Promega UK Ltd, Delta House, Southampton Science Park, Southampton, Hampshire SO16 7NS, UK). Unless otherwise stated,

the DNA template used in PCR was from a sample of wheat DNA (20 ng μL^{-1}) extracted from leaf tissue of Cadenza variety wheat.

Leaf DNA extraction

DNA was extracted from wheat leaf tissue using the following method. 400 µL DNA buffer (200 mm Tris-HCl (pH8), 25 mm EDTA, 250 mm NaCl, 0.5% SDS) was added to leaf tissue in a 1.5 mL Eppendorf tube and macerated with a centrifuge tube pestle. 135 µL 5 m potassium acetate was added, the tube vortexed and incubated on ice for 10 min. Microfuged at full speed for 15 min. Supernatant transferred into fresh tube, 0.8 volumes -20°C isopropanol added and mixed gently before incubating at -20°C for 30 min. Microfuged at full speed for 15 min. Supernatant discarded and pellet washed with 600 µL chilled 70% ethanol. Microfuged at full speed for 5 min. Supernatant discarded and pellet washed with 300 µL chilled 100% ethanol. Microfuged at full speed for 5 min. Supernatant discarded, then pellet dried and dissolved in H_2O.

Polymerase chain reaction

PCRs were performed using an Eppendorf Mastercycler PCR machine. Each reaction tube contained a total reaction mixture of 20 µL composed of 12.8 µL H_2O, 2 µL 10 × PCR buffer, 0.6 µL 50 mm MgCl2, 1.6 µL 2.5 mm dNTPs, 0.4 µL forwards primer, 0.4 µL reverse primer, 2 µL DNA template, 0.2 µL Taq polymerase. The general PCR programme used was 94°C for 5 min, followed by a denaturing, annealing, amplification cycle (repeated 35 times) of 94°C, 60°C (for non-gradient PCR), 72°C. Once complete, this repeated cycle was followed by 72°C for 10 min prior to removal of tubes from machine and storage at 4°C.

Agarose gels

The electrophoresis gels were 2% agarose, made up with 50 mL 0.6 × TBE buffer (5 × TBE buffer: 53 g Tris base, 27.5 g boric acid, 10 mm EDTA, made up to 1 L with H_2O). 5 µL 10 mg mL^{-1} ethidium bromide was mixed into the buffer once the agarose had been dissolved and the liquid had begun to cool. Gels were left to solidify in a microgel former before being placed in a microgel bath and submerged in 0.6 × TBE buffer. 2 µL loading buffer (10 mL loading buffer: 25 mg xylene cyanol, 25 mg bromophenol blue, 3 mL glycerol, made up to 10 mL with H_2O) was added to 10 µL of PCR product in each of the experiment wells. 200 µL of 50 bp DNA step-ladder (Sigma-Aldrich Ltd, The Old Brickyard, New Road, Gillingham, Dorset SP8 4XT, UK) was added to 40 µL loading buffer. 5 µL of the mixture was added to each marker well. Once PCR products and marker had been loaded, gels were run at 100V then photographed under ultraviolet light.

E. coli *transformation and culture*

A sample of pHMW-adh-nos vector was obtained from Dr Huw Jones (Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK). Competent DH5-α *Escherichia coli* cells were transformed using this sample of vector. 100 µL competent cells were added to 5−10 ng of plasmid DNA and an empty control tube. The tubes were mixed gently then placed on ice for 30 min. Tubes were placed in a 37°C water bath for 2 min. 900 µL LB media was added to each tube, and tubes were kept at 37°C for a further 30 min. 100 µL and 10 µL were taken from the transformed tube and spread onto separate LB+Ampicillin plates (LB+Ampicillin plates: 100 mL LB agar microwaved until liquid. Once sufficiently cooled, 100 µL 100 mg mL^{-1} Ampicillin was added and mixed. 25 mL was poured into each of three sterile Petri dishes and left to cool). 100 µL of the control tube was transferred onto the third plate. After 5 min the plates were inverted and incubated at 37°C for 16 h.

Transformed, Ampicillin-selected colonies were then visible, of which two were used to inoculate LB+Ampicillin media (LB+Ampicillin media: 10 mL sterile LB media mixed with 10 µL 100 mg mL^{-1} Ampicillin) in two tubes which were incubated at 37°C in a shaking incubator. Plasmid

was recovered from the culture using a GeneJET™ Plasmid Miniprep Kit (Fermentas GmbH, Opelstrasse 9, 68789 St Leon-Rot, Germany). A sample of the remaining culture was used to make a glycerol stock (1.5 mL culture added to 0.5 mL 60% glycerol in cryotube, vortexed, frozen in liquid N_2 and stored at -70°C), from which further *E. coli* cultures could be made when more plasmid was required.

Cloning of construct

Bovine serum albumin, Calf intestinal alkaline phosphatise, T4 DNA ligase enzyme and *Bam*HI and *Bgl*II restriction enzymes were acquired from Promega (Delta House, Southampton Science Park, Southampton SO16 7NS, UK). Digestion and ligation of insert and vector were performed using the supplier's instructions. Negative controls used were: no enzyme digest (insert & vector), no enzyme ligation, no vector ligation, no insert ligation. The ligated construct was used to transform competent DH5-α *E. coli* cells. During the colony screening PCR 2 combinations of primers were used for each colony to determine insert presence and number. Eight colonies were selected for further screening using six different PCR primer combinations for each. Four of those colonies contained plasmid that had ligated correctly, of which one was sequenced.

Wheat seed collection

Wheat plants (*var*. Cadenza) were grown in glasshouses (glasshouse conditions: venting at 22°C, day heating at 20°C, night heating at 18°C, 16 h day length, supplemental lighting outside of British Summer Time) at Sutton Bonington campus of Nottingham University (College Road, Sutton Bonington LE12 5RD, UK). The date when each ear reached anthesis was noted using the decimal code devised by Zadoks *et al*. (1974). Ears were said to have anthesed when they passed 61. Seeds were harvested at 4, 7, 10, 14, 17, 21, 28 and 35 days post anthesis (dpa) and frozen in liquid nitrogen before being stored at -70°C. Some seeds were dissected to obtain the endosperm after harvesting and before freezing.

RNA extraction

RNA was extracted from wheat seed tissue using a modified version of the method used by Chang *et al*. (1993). Following RNA extraction, the RNA was analysed by Nanodrop™ 1000 spectrophotometer and Agilent™ 2100 bioanalyser, and stored at -70°C.

Results

Phylogenetic analyses were carried out using Rab protein and nucleotide sequence data from *Arabidopsis thaliana, Brachypodium distachyon*, rice, wheat and tobacco. Selected sequences were aligned together and phylograms were generated from those alignments in order to discern relationships between individuals and groups of Rab sequences.

Rab coding sequences from wheat were phylogenetically compared (Fig. 1). The pattern seen is generally similar to that seen in *Arabidopsis* and other plants. When comparing the genes of wheat and *Arabidopsis* (data not shown) each clade tends to group together in the tree, whilst subgroups within them often show more similarity between sequences of their own species rather than the equivalent sequence(s) belonging to the other organism. The *RabD* clade is a case in point - there appears to be a reasonable level of similarity between *RabD2a* sequences from both wheat and *Arabidopsis*, however the wheat *RabD1* sequences are fairly dissimilar to the *Arabidopsis RabD1* in relation to the rest of the clade (see Fig. 2). The wheat *RabD2* gene Ta.54881 also shows relatively little similarity to the *Arabidopsis RabD2* members. The explanation for this could be the evolutionary distance between *A. thaliana* and *T. aestivum*, or possibly the effects of hexaploidy (e.g. silencing, pseudogenes, redundancy) in wheat compared to the diploid genome of *Arabidopsis*.

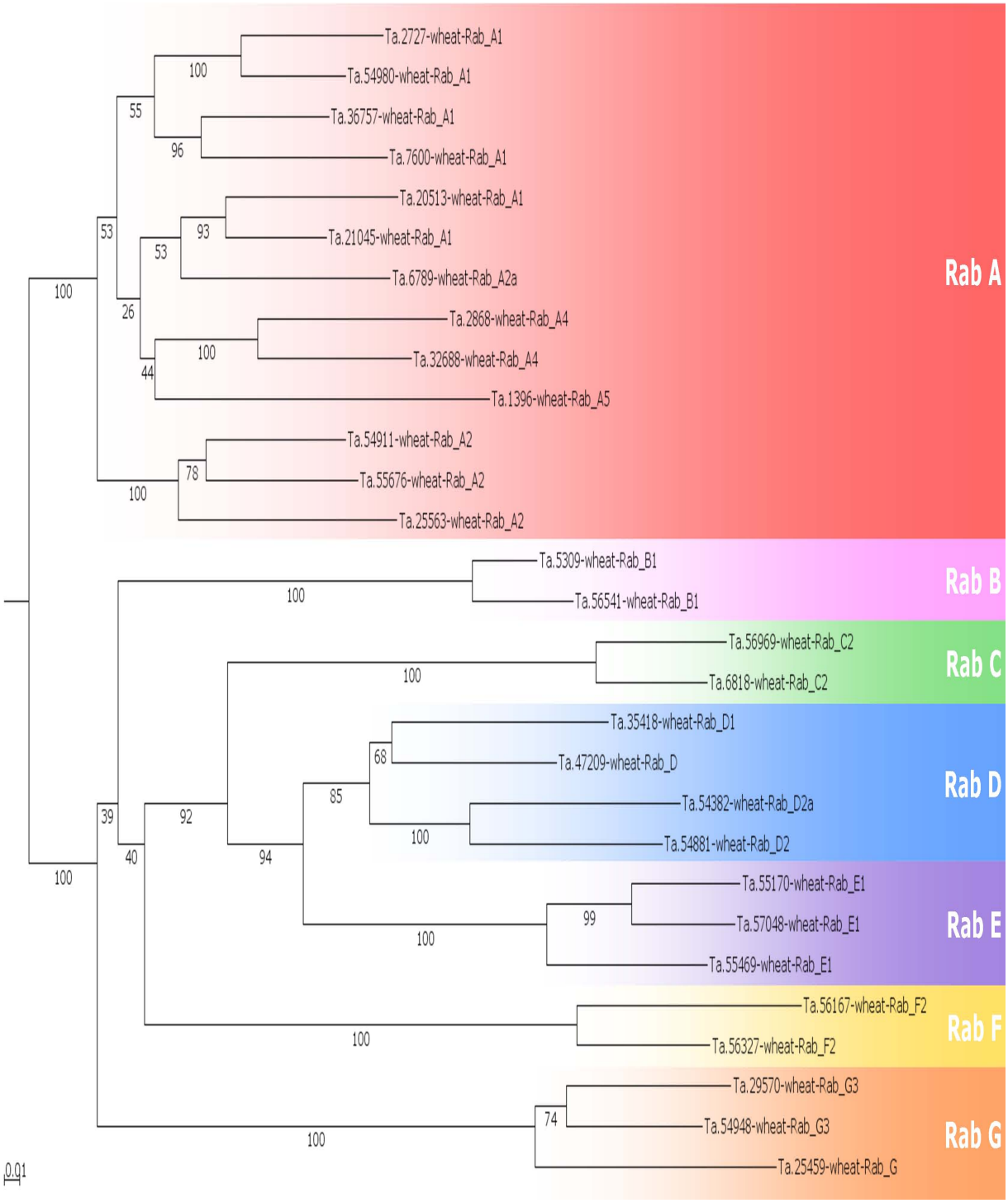

Fig. 1. Phylogram containing *Rab* coding sequences from wheat. *Rab* clades are indicated on the right. Labels include: unigene name, organism, tentative classification.

The phylogram shown in Fig. 2 shows the available *RabD* coding sequences from *Arabidopsis*,rice, wheat, *Brachypodium* and the tobacco Rab targeted in an earlier study by Di Luccia *et al*. (2005). While *RabD2a* and to a certain extent *RabD1* sequences appear to be conserved within their own subgroups, the *Arabidopsis RabD2b* (AT5G47200) and *RabD2c* (AT4G17530) appear to be more similar to each other than any *RabD2* sequences of other species. This could make it difficult to classify *RabD2* sequences that don't appear homologous to *RabD2a*.

The gene encoding the tobacco Rab D2a protein included in this phylogram was used to silence the native homologue in durum wheat (*Triticum durum*) and was shown to influence protein trafficking between the ER and the Golgi (Di Luccia *et al*., 2005). A similar effect might be achieved by reducing the expression of the same isoform in bread wheat.

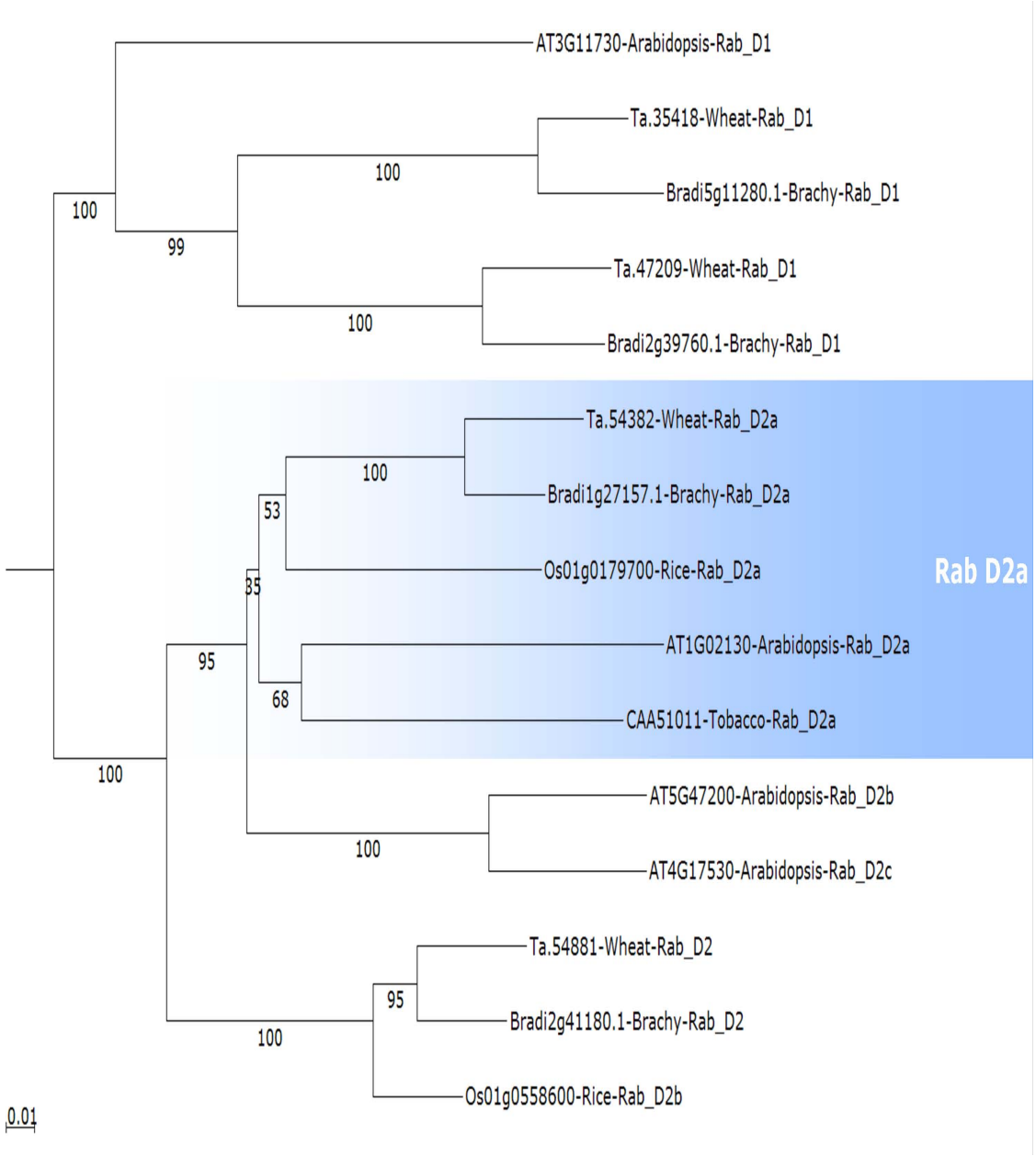

Fig. 2. Phylogram comparing the RabD coding sequences of wheat, *Brachypodium*, rice, *Arabidopsis* and tobacco. *RabD2a* homologues are highlighted in blue. Labels include: identifier, organism, tentative classification.

Discussion

The Rab GTPases of wheat share significant sequence similarity with Rabs of other plants in which there is a better understanding of the cell trafficking system. This information may be used to aid investigation into the specific roles Rab GTPases play in the transport and storage of important seed storage proteins during wheat seed development.

Transgenic hexaploid wheat plants have been generated at Rothamsted Research using an RNAi construct designed to reduce expression of *Ta.54382*. The final generation of homozygous transgenic plants should be ready for harvest in late 2011.

Silencing the *Rab1B* gene in durum wheat changed the functional properties of the grain and resulted in improved gluten quality (Di Luccia *et al*., 2005). The transport of gluten proteins within the secretory system was thought to have been altered by down-regulating the trafficking step from the ER to the Golgi. Down-regulating the expression of one or more *RabD* genes may

allow us to manipulate the ratio of different types of seed storage protein and the way they are stored. This could ultimately alter, and possibly improve the rheological properties of the grain for the purpose of bread making.

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