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Interaction network of proteins associated with abiotic stress response and development in wheat

Guylaine Tardif · Ndjido A. Kane · Hélène Adam · Louisette Labrie · Geneviève Major · Patrick Gulick · Fathey Sarhan · Jean-François Laliberté

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Abstract Wheat is the most widely adapted crop to abiotic stresses and considered an excellent system to study stress tolerance in spite of its genetic complexity. Recent studies indicated that several hundred genes are either up- or down-regulated in response to stress treatment. To elucidate the function of some of these genes, an interactome of proteins associated with abiotic stress response and development in wheat was generated using the yeast two-hybrid GAL4 system and specific protein interaction assays. The interactome is comprised of 73 proteins, generating 97 interactions pairs. Twenty-one interactions were confirmed by bimolecular fluorescent complementation in *Nicotiana benthamiana*. A confidence-scoring system was elaborated to evaluate the significance of the interactions. The main feature of this interactome is that almost all bait proteins along with their interactors were interconnected, creating a spider web-like structure. The interactome revealed also the presence of a ''cluster of proteins involved in flowering control'' in three- and four-protein interaction loops.This network provides a novel insight into the complex relationships among transcription factors known to play central roles in vernalization, flower initiation and abscisic acid signaling, as well as associations that tie abiotic stress with other regulatory and signaling proteins. This analysis provides useful information in elucidating the molecular mechanism associated with abiotic stress response in plants.

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G. Tardif · L. Labrie · J.-F. Laliberté (✉)
Institut Armand-Frappier, Institut national de la recherche scientifique, 531 boulevard des Prairies, Laval, Québec, Canada, H7V 1B7
e-mail: jean-francois.laliberte@iaf.inrs.ca

N. A. Kane · H. Adam · G. Major · F. Sarhan
Département des sciences biologiques, Université du Québec à Montréal, CP 8888, Succursale Centre-ville, Montréal, Québec, Canada, H3C 3P8

P. Gulick
Department of Biology, Concordia University, 7141 Sherbrooke W, Montréal, Québec, Canada, H4B 1R9

Introduction

Plants have evolved to survive under a wide range of environmental conditions and they display large genetic variation for tolerance to stresses such as freezing, drought, salinity, heavy metals, high light, and increased atmospheric CO_2. Tolerant plants cope with harsh environmental conditions through adaptive mechanisms that are genetically programmed and that result in the production of a wide array of substances needed to protect the plants and ensure their survival. Wheat (*Triticum aestivum*) is one of the two major cereals world wide, with the production of over 627 million tones in 2004 (http://faostat.fao.org/). It is grown over a large range in latitudes under both rain fed and irrigated conditions and thus in conditions subjected to environmental stresses. Bread wheat is among the most cold tolerant crop species and winter wheat cultivars are markedly more freezing tolerant than spring cultivars. The control of flower induction is a key element that distinguishes these classes of cultivar types and is intimately linked to cold tolerance.

The signalling pathways that regulate flowering, vernalization and stress responses have been extensively studied in the model species *Arabidopsis thaliana*. Numerous mutants that affect flower induction have been characterised and relationships between components of signalling and regulatory pathways have been determined by epitasis. In wheat, key genes regulating flower induction in relation to vernalization, VRN1/TaVRT1, VRN2 and TaVRT2 have been identified recently (Danyluk et al. 2003; Yan et al. 2004; Kane et al. 2005). These genes belong to transcription factor gene families and have homologs in *A. thaliana*. Though members of these gene families have been shown to play central roles in the regulation of flowering in *A. thaliana*, it is not possible to determine orthology relationships between the wheat and *A. thaliana* genes by sequence similarity alone. It is also likely that the signalling pathways in the two species are not identical, for example the genes controlling the requirement for vernalization in wheat and *A. thaliana* are not orthologous (Yan et al. 2003, 2004).

Genomic studies in crop species have facilitated all aspects of the study of their molecular biology. Large-scale genomic sequencing in rice and *A. thaliana* and EST sequencing in other important species have accelerated the identification of genes based on sequence similarity to gene of known function. Large-scale gene expression studies by microarray analysis have identified many genes that are associated with the environmental stress responses. Protein–protein interaction studies can give critical insight into the components of signalling and regulation of the cold stress response and flower induction. Such an approach is especially important in crop species in which extensive mutant stocks are not available and in which it is difficult to establish gene interaction relationships through epistasis. It can delineate components of signalling and regulation networks by the identification of the direct interaction partners of regulatory proteins and identify proteins that may not be identified by changes in their mRNA levels.

Light perception and temperature sensing are key elements in the plants response to environmental stress. Secondary messengers such as Ca^{2+}, calmodulin (CaM), cAMP, cGMP, cADP-ribose, inositol 1,4,5-triphosphate (IP_3) and reactive oxygen species (ROS) have been implicated in signalling in the stress response. These secondary messengers relay the stress signal by the intermediary of transducer kinases and phosphatases to appropriate transcription factors, which act as positive or negative expression regulators of target genes. This re-programming of gene expression is needed for cell damage repair and prolonged abiotic stress protection [for reviews on this subject, see (Mahajan and Tuteja 2005; Xiong et al. 2002)].

Recently, there has been a number of extensive studies using microarrays of gene regulation during abiotic stress of *A. thaliana* (Hannah et al. 2005; Lee et al. 2005), rice (Rabbani et al. 2003; Yamaguchi et al. 2004), potato (Rensink et al. 2005) and wheat (Gulick et al. 2005). These studies indicated that several hundred genes are either up- or down-regulated in response to stress. A large number of these genes encode regulatory factors such as protein kinases, transcription factors, ubiquitin ligases, GTP and calcium binding proteins or are involved in chromatin modification or post-transcriptional regulation. Several genes involved in the biosynthesis of plant hormones, such as abscisic acid (ABA), gibberellic acid and auxin, are also regulated by stress. However, not all abiotic stress-related gene products may be transcriptionally regulated, and the co-ordinated regulation of genes is only suggestive of interactions.

Protein–protein interactions are likely to play an important role in response to abiotic stress, in the signal transduction cascade for example. Therefore, compiling the interaction network will provide a novel perspective on how cells perceive and transduce stress signals to trigger the genetic system responsible for appropriate plant response. The yeast two-hybrid system is a powerful tool for the identification of protein associations that can be applied to high-throughput detection of interactions across the entire proteome of an organism. The generation of accurate cellular protein interaction networks is an ongoing process. Proteome-wide studies for model organisms such as *Helicobacter pylori* (Rain et al. 2001), *Saccharomyces cerevisiae* (Ito et al. 2001; Uetz et al. 2000), *Caenorhabditis elegans* (Li et al. 2004) and *Drosophila melanogaster* (Giot et al. 2003), in addition to *Homo sapiens* (Rual et al. 2005; Stelzl et al. 2005), have been performed. However, there are few studies on protein interaction mapping in plants. One study showed a densely connected network of interactions between and within family members of the 3-aa loop extension (TALE) homeodomain proteins (Hackbusch et al. 2005). The interaction map of the *A. thaliana* MADS-box transcription factors has been investigated and revealed regulatory loops providing links between flower organ development and floral induction (de Folter et al. 2005). The hallmark of these networks is that practically all proteins are linked to each other, a characteristic of small-world networks. Another property is that the number of links per protein is non-uniform (i.e. scale-free), with the great majority of proteins with only a few connections along with the

presence of ''hubs'' in which proteins are highly connected. This scale-free topology is linked to the robustness of interaction networks, being largely insensitive to random removal of single proteins but sensitive to removal of ''hubs''. Although large interactome maps establish only ''scaffold'' information of protein–protein interactions without describing the dynamics of interaction, they nevertheless provide unique resources for further functional studies and the identification of key proteins.

The goal of this study is to initiate an interactome of proteins associated with abiotic stress response and development in wheat. A certain number of proteins known to play an important role in these processes were chosen as initial baits for the screening of interactors coded by cDNA libraries, or were directly tested with specific proteins whose candidacy for interaction was suggested by investigations in model species. In addition, selected putative interactors were subsequently reconfigured as baits for a second round of screening. Certain interactions were confirmed in planta by bimolecular fluorescent complementation (BiFC). The significance of the generated interactome of 73 proteins, with 97 links between them is discussed.

Materials and methods

Yeast growth conditions

The *S. cerevisiae* strains used in this study are Y187 *MAT* α and AH109 *MATa*. Yeast cells were grown on standard YPD [1% (w/v) yeast extract (Difco), 2% (w/v) Bacto peptone (Difco), 2% (w/v) dextrose] or YNB [0.67% (w/v) yeast nitrogen base without amino acids (Difco) supplemented with the appropriate amino acids (Sigma) and containing 2% dextrose or 1% raffinose/2% galactose]. The yeast strains were transformed using the modified lithium acetate method (Gietz and Woods 2001).

Wheat cDNA libraries

For the cold acclimation and developmental cDNA library, seeds of *Triticum aestivum* L. cv Nostar were germinated in water-saturated vermiculite for 7 days under an irradiance of 200 μmol m^{-2} sec^{-1}. The temperature was maintained at 20°C with a 15-h photoperiod under a relative humidity of 70%. At the end of this period, non-acclimated plants were sampled and frozen. Cold acclimation was performed by subjecting germinated seedlings to a temperature of 4°C with a 12-h photoperiod for one, 23 and 53 days. RNA from aerial parts (i.e. crown and leaf) were isolated using the Tri reagent (Sigma) from seven day non-acclimated plants and 1, 23, and 53 day cold-acclimated plants, and pooled.

For the dehydration stress cDNA library, seeds were germinated in water-saturated vermiculite for 7 days under an irradiance of 200 mol m^{-2} sec^{-1}. The temperature was maintained at 20°C with a 15 h photoperiod under a relative humidity of 70%. At the end of this period, plants were removed from vermiculite and incubated at 20°C on the table without water for 1, 2, 3 and 4 days and then sampled. In parallel, seeds were germinated in a water-saturated mix (50% black earth and 50% ProMix) for seven days under an irradiance of 200 mol m^{-2} sec^{-1}. The temperature was maintained at 20°C with a 15 h photoperiod under a relative humidity of 70%. After this period watering of plants was stopped. Four time points were sampled during a two week period; the first after wilting was observed and the last two weeks later, consisting of live crown and stem tissue (leaf tissue was yellow and not included in sampled material). RNA from aerial parts (i.e. crown and leaf) from these time points were isolated using the Tri reagent (Sigma) and pooled.

The ''SuperScript plasmid System'' (Invitrogen) was used for cDNA synthesis. The library was produced in pEXPAD-502 (Invitrogen).

Two-hybrid analysis

Two-hybrid analyses using the dehydration library were performed with the GAL4 yeast two-hybrid system. The dehydration library was sub-cloned in pDEST22 prey vector by Gateway cloning and introduced into *S. cerevisiae* strain AH109 (*MAT*a *trp1, leu2* which contains *ADE2, HIS3, lacZ* and *MEL1* reporter genes, each of which uses a distinct GAL4-responsive promoter). Bait-coding cDNAs were PCR amplified with specific primers (see supplementary material) and cloned in pDEST32 bait vector (Invitrogen) by homologous recombination in *S. cerevisiae* strain Y187 (*MAT*α *trp1*, *leu2*, which contains the *lacZ* and *Mel1* reporters under the control of two distinct GAL4-responsive promoters). Yeast cells were grown and transformed as previously described (Gietz and Woods 2001). Diploid cells between Y187 (bait) and AH109 (wheat cDNA library containing 10^6 clones) were obtained by conjugation. Yeast colonies containing putative interactors were selected on medium containing SD/Galactose/Raffinose without adenine, leucine, tryptophane and uracil, supplemented with 10 mg/l of 5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside (X-α-Gal) (VWR) Blue colonies were

picked after 6 days at 30°C. The prey-coding genes were identified by sequencing. Interaction was confirmed by a second round of two-hybrid analysis with β-Galactosidase (*Lac*Z) assay on solid medium (Dumay et al. 1999).

The cold acclimated and development library was screened according to the Gateway ProQuest Two hybrid system instructions (Invitrogen) - 2,5 × 10^6 clones were screened using this library.

The interaction data was displayed using the yEd Graph editor (http://www.yworks.com/)

Agroinfiltration

The backbone of the fusion vector used for Bi-molecular fluorescence complementation was pGreen-0029 vector (Hellens et al. 2000), which was modified in the following manner. The multiple cloning site (MCS) was removed by digestion with *Kpn*I and *Not*I followed by filling up of overhangs and ligation. The 35S cassette (http://www.pgreen.ac.uk/JIT/pGreen0000_fr.htm) was then inserted into the *Stu*I site within the right border of the T-DNA. The 2μTRP1 gene for yeast DNA replication was amplified by PCR using specific primers (see supplementary material) and introduced by homologous recombination in the *Hpa*I-linearized vector following transformation of yeast strain Y187. The Ntgfp5 (1–471) and Ctgfp5 (472–714) were amplified from pBin-mgfp5-ER (Haseloff et al. 1997) with specific primers (see supplementary material), and inserted into the *Sma*I-linearized above described modified pGreen-0029 vector by homologous recombination. The resulting plasmids were identified as pGreen-35S-NtGFP5 or pGreen-35S-CtGFP. Genes of interest were amplified by PCR using specific primers (see supplemental data) and introduced into the yeast strain Y187 for homologous recombination with the appropriate BiFC vector. Each construct was analysed by PCR and sequenced.

Agrobacterium tumefaciens strain AGL1 transformed with the appropriate BiFC vector was grown at 30°C in L-broth supplemented with 50 g ml^{-1} of kanamycin and 50 g ml^{-1} of ampicillin to stationary phase. Bacteria were sedimented by centrifugation at 5000 *g* for 15 min at room temperature and resuspended in 10 mM $MgCl_2$ and 150 g ml^{-1} acetosyringone. Cells were left in this medium for 3 h to over night at room temperature. The abaxial air space of leaves from four-week-old *Nicotiana benthamiana* plants were infiltrated (Voinnet et al. 2003). The P0 protein of beet western yellows virus was used to suppress posttranscriptional gene silencing. Co-infiltration of *Agrobacterium* strains containing the BiFC constructs and the P0 silencing plasmid was carried out at OD_{600} of 0,5: 0,5: 0,5. Epidermal cell layers of tobacco leaves were assayed for fluorescence 3–5 days after infiltration. *N. benthamiana* plants were incubated in environmental growth chamber under long days (16 h light/8 h dark) at 20°C. Wide field fluorescence imaging was carried out on Nikon E800 upright equipped with a Radiance 2000 BioRad laser. Excitation was with BioRad laser at 515 ± 15 nm longpath. Image analysis was carried out with Photoshop 7.0.

Results and discussion

Two-hybrid screen

The protein–protein interaction map was generated using the yeast two-hybrid GAL4 system using a combination of library screens and specific protein interaction assays. Proteins that were selected as baits for the initial screening of a wheat cDNA library are listed in Table 1. These proteins are transcription factors or signal transduction components that have been shown to be associated with stress responses, vernalization and/or flower development. In each case, baits were tested for auto-activation prior to screening. Conjugation mating between the yeast strain harbouring the bait plasmid with yeasts harbouring the prey cDNA plasmids was performed. Only colonies positive for all three reporter genes (i.e. growth on plates lacking adenine as well as histidine, and expression of x-alpha Gal activity) were retained. Positive colonies were picked and the prey genes were sequenced. Prey plasmids were isolated and tested in a series of control two-hybrid interaction assays. When available, the full-length cDNA of the putative interactor was preferentially used. Only preys showing beta-galactosidase activity with the bait and not with control proteins were retained. Ten preys having a high potential for involvement in abiotic stress response or development were reconfigured as baits for another round of library screening. Equally, specific protein interaction assays were performed using the same experimental criteria as for a library screen.

The interaction data for the library screening and specific protein interaction is shown in Table 2. About 20 proteins were configured as baits (i.e. 10 proteins listed in Table 1 and 10 resulting from preys that were reconfigured as baits) and 63 preys were identified, generating 97 interaction pairs. Of those interaction pairs, 18 were from specific protein–protein interactions. The biological significance of the interactions was evaluated by searching the PubMed and Google

Table 1 Proteins used as initial bait in two-hybrid screening

Initial bait	Evidence for involvement in abiotic stress and/or development	Ref.
TaVRT-1/VRN-1 *AY280870*	MADS-box transcription factor; member of the AP1 subfamily; associated with vegetative to reproductive transition in cereals following vernalization.	Danyluk et al. 2003
TaVRT-2 *DQ022679*	MADS-box transcription factor; member of the StMADS-11 subfamily of flowering repressors; regulated by vernalization and photoperiod in wheat.	Kane et al. 2005
TaTIL *AAL75812*	True lipocalin; transcript induced by low temperature; possible role against stress damage.	Frenette-Charron et al. 2002; 2005
TaCHL *CK159974*	Chloroplastic lipocalin; transcript induced by low temperature; repressed by heat shock; possible role against stress damage.	Frenette-Charron et al. 2005
TaFCA-A1 *AAP84415*	Homologue to the barley abscisic acid-binding protein ABA-P1 and to *A. thaliana* FCA; involved in flowering.	Razem et al. 2006
Small Ran-related GTP-binding protein *AF433653*	Involved in signal transduction; transcript regulated differently in spring and winter wheat.	Gulick et al. 2005
TaGB1 (GTP binding protein) *DQ489316*	Involved in signal transduction; transcript regulated differently in spring and winter wheat.	Gulick et al. 2005
Inorganic pyrophosphatase *CD862876*	Transcript regulated differently in spring and winter wheat.	Gulick et al. 2005
Receptor-like protein kinase *AF085168*	Involved in signal transduction; transcript regulated differently in spring and winter wheat.	Gulick et al. 2005
Calcium binding EF-hand protein *DR733548*	Involved in signal transduction; transcript regulated differently in spring and winter wheat.	Gulick et al. 2005

Scholar literature databases for co-occurrence of prey and bait protein names, or for association of prey protein names with abiotic stress, signal transduction or gene expression regulation. None of the interactions were previously reported in the literature, as expected from the paucity of protein interaction data for wheat. For 16 interaction pairs, there was previous evidence for similar association in plant or animal cells. For example, MADS-box transcription factors have been shown to form homo- and heterodimers (de Folter et al. 2005), and tubulin interaction with phospholipase C and kinases has been reported (Popova et al. 1997; Carman et al. 1998). In several instances, the prey protein was associated with abiotic stress response (13 occurrences). Equally, many prey proteins were involved in signal transduction (e.g. immunophilin, G protein alpha subunit, MAPK phosphatase; 12 occurrences), or gene expression regulation (e.g. TaHD, VRN-2, Elongin C, RNA polymerase II 36 kDa subunit; 16 occurrences).

It is worthy to note that the library screening using MADS-box proteins as bait did not identified other MADS-box proteins as prey. The most likely explanation is that the cDNAs encoding these factors are of very low abundance in the libraries. Another possibility is some interaction might be mediated by another protein, or DNA. Finally, some MADS-box proteins may require post-translational modifications for interaction, which do not take place in yeast.

Bimolecular fluorescent complementation experiments (BiFC)

Selected interaction pairs uncovered by the yeast two-hybrid screen and by specific protein interaction assay were further tested for in planta association by BiFC (Bracha-Drori et al. 2004; Walter et al. 2004). In this assay the green fluorescent protein (GFP) is split into two non-overlapping N-terminal (GNter) and C-terminal (GCter) fragments. The GNter fragment is fused to the N-terminal end of one binding protein, while the GCter fragment is fused to the C-terminal end of the corresponding partner. Both fusions are expressed concomitantly in *N. benthamiana* by agro-infiltration, and reconstitution of a fluorescing GFP chromophore examined by confocal microscopy 4 days later. The interactions selected for BiFC assays were chosen a priori for their presumed biological significance and in several cases were based on some prior knowledge of interaction in signalling pathways. About 21 interaction pairs were tested, and all proved to be positive. Table 3 lists these interactions, as well as the predominant cellular site of interaction (i.e. cytoplasm and/or nucleus). Each protein fusion was tested against a control non-interacting protein fusion to ascertain that the observed fluorescence was not the result of non-specific interaction. Figure 1 provides representative confocal micrographs of interacting pairs that were

Table 2 Protein–protein interaction data

Bait	Prey	Published co-occurrence of prey with bait, with abiotic stress, signal transduction or gene expression regulation	Ref.
Calcium binding EF-hand protein[a] *DR733548*	TaGA1 (G protein alpha subunit)[c] *AB090158*	Heterotrimeric G protein are involved in several signal transduction pathways.	Hossain et al. 2003
Elongin[b] *CV765696*	Histone H2B[c,d] *P27807*		
	60S Ribosomal protein L9[c] *BQ806898*		
	Translation elongation factor 1-alpha[c] *AF479046*		
Immunophilin[b] *X86903*	Metallothionein-like protein[c] *L11879*	Metallothionein functions in both metal chaperoning and scavenging of reactive oxygen species.	Mir et al. 2004
	Pathogenesis related protein 1[c,d] *AF384143*	Involvement in cold adaptation is inferred from activation of the pepper basic PR-1 gene promoter after exposure of plant at 4°C.	Hong et al. 2005
	Peroxidase[c] *X56011*	Injury to plants imposed by cold exposure is associated with oxidative damage at the cellular level. Correlation between active oxygen detoxifying enzymes and chilling tolerance has been noted.	Yoshimura et al. 2004
Inorganic pyrophosphatase[a] *CD862876*	Cold acclimation induced protein ESI2-1[c] *AY666013*	Gene expression is induced during cold acclimation.	Gulick, unpublished
	Glycosyltransferase[c] *AJ969052*	Accumulation of zeatin *O*-glycosyltransferase in *Phaseolus vulgaris* and *Zea mays* was observed following cold stress.	Li et al. 2000
	60S Ribosomal protein L9[c] *BQ806898*		
Lipid transfer protein 3[b] *AY226580*	*S*-adenosylmethionine decarboxylase[c] *CK209496*	Differential expression of transcript upon low temperature treatment has been reported for a cold resistant rice genotype.	Pillai and Akiyama 2004
	Glutathione *S*-transferase[c,d] *CV781641*	Abiotic stress alters transcript profile and activity of glutathione *S*-transferase.	Anderson and Davis 2004
	Polygalacturonase[c] *CA638207*	Two *Brassica napus* polygalacturonase inhibitory protein genes are expressed at different levels in response to biotic and abiotic stresses.	Li et al. 2003
	Unknown protein 3[c] *DR736877*		
	Vacuolar targeting receptor[c,d] *AAF80450*		
β-Ketoacyl-acyl carrier protein synthase[b] *DR740728*	Phosphoinositide-specific phospholipase C[c] *DR741524*	Changes in phospholipase C activity affect the expression of a large number of cold regulated genes in *A thaliana*.	Vergnolle et al. 2005
Pathogenesis related protein 1[b] *AJ007348*	Fructan 1-exohydrolase[c,d] *CAD56806*	Expression analysis of a chicory fructan 1-exohydrolase gene revealed complex regulation by cold.	Michiels et al. 2004
	Glycosyltransferase[c] *AJ969052*	Accumulation of zeatin *O*-glycosyltransferase in *Phaseolus vulgaris* and *Zea mays* was observed following cold stress.	Li et al. 2000
	Cold acclimation induced protein ESI2-1[c] *AY666013*	Gene expression is induced during cold acclimation.	Gulick, unpublished
	Unknown protein 1[c] *CD931572*		
	Wheat etiolated seedling root[c] *DN829250*		

Table 2 continued

Bait	Prey	Published co-occurrence of prey with bait, with abiotic stress, signal transduction or gene expression regulation	Ref.
Receptor-like protein kinase[a] *AF085168*	Phosphatidylglycerol specific phospholipase C[c] *CK206104*	Changes in phospholipase C activity affect the expression of a large number of cold regulated genes in *A. thaliana*.	Vergnolle et al. 2005
	α-Tubulin 3-3[c,d] *DQ435660*	Microtubules participate in the cold stress response and in adaptation to low temperatures; binding and phosphorylation of tubulin by G protein-coupled receptor kinases have been reported.	Nyporko et al. 2003; Carman et al. 1998
	unknown protein 2[c] *CV779217*		
Small Ran-related GTP-binding protein[a] *AF433653*	Cold acclimation induced protein ESI2-1[c] *AY666013*	Gene expression is induced during cold acclimation.	Gulick, unpublished
	Unknown protein 1[c] *CD931572*		
Ta31K05[b] (SVP-like *St*MADS-11) *CV762849*	Pathogenesis related protein 1.1[c,d] *AJ007348*	Very similar to *AF384143*. Involvement in cold adaptation is inferred from activation of the pepper basic PR-1 gene promoter after exposure of plant at 4°C.	Hong et al. 2005
Ta45G05[b] AGL12 *DR741518*	Fructan 1-exohydrolase[c,d] *CAD56806*	Expression analysis of a chicory fructan 1-exohydrolase gene revealed complex regulation by cold.	Michiels et al. 2004
	α-Tubulin 3-3[c,d] *DQ435660*	Microtubules participate in the cold stress response and in adaptation to low temperatures.	Nyporko et al. 2003
	Fructose-biphosphate aldolase[c] *BE216994*		
	Protein tyrosine phosphatase[c,d] *CV772785*	A tyrosine-specific protein phosphatase is encoded by a stress-responsive gene in *A. thaliana*.	Xu et al. 1998
	Thiamine biosynthesis protein[c,d] *CV763653*		
	Thiol protease aleurain[c] *CV778706*		
Ta57H08[b] (SVP-like *St*MADS-11) *CV769487*	Calmodulin TaCaM3-1[c,d] *AAC49584*	Calmodulin(CaM) is an important intermediate of calcium-mediated signal transduction; binds also wheat immunophilin.	Liu et al. 2003; Kurek et al. 2002
	Ferrodoxin-NADP(H oxidoreductase[c,d] *CAD30025*	Scavenger of free oxygen radicals.	Krapp et al. 1997
	Plastid ribosomal protein CL9[c] *AAM92711*		
	Sigma related factor[c,d] *NP 197800*		
Ta73C21[b] (AGL14 TM3/SOC1) *CV772064*	Plastocyanin precursor SGT1[c] *CA625601*		
TaCHL (chloroplast lipocalin)[a] *CK159974*	Endopeptidase ATP-B chain C[c,d] *CV780642*		
	Glu-tRNA amidotransferase A[c,d] *DR738631*		
	High mobility protein[c,d] *CAA77641*		
	β-Ketoacyl-acyl carrier protein synthase[c,d] *DR740728*	Involved in fatty acid synthesis.	Abbadi et al. 2000
	Lipid transfer protein 3[c,d] *AY226580*	A lipid transfer protein gene was shown to be differentially regulated by abiotic stress and ABA.	Wu et al. 2004
	40S ribosomal p525[c] *CD930290*		
	Rubisco small unit[c] *X00235*		

Table 2 continued

Bait	Prey	Published co-occurrence of prey with bait, with abiotic stress, signal transduction or gene expression regulation	Ref.
TaFCA-A1[a] (Abscisic acid-binding protein like) *AAP84415*	Lipoxygenase[d] *U32428*	Lipoxygenases are involved in stress responses and are modulated by ABA.	Melan et al. 1993
	MAP kinase phosphatase[c] *AJ606016*	Negative regulator of mitogen-activated protein kinase, which is involved in ABA signal transduction.	Xiong et al. 2002; Meskiene et al. 1998
	Solanum pollinated pistil (SPP30)[c] *AL819796*	Coded by conserved gene in evolutionarily distant organisms; predicted to have an important role in development.	Lantin et al. 1999
	Sucrose-6F-phosphate phosphohydrolase[c] *AY029159*	Pulls the sucrose synthesis reaction pathway in the direction of net sucrose synthesis; sucrose is an osmoprotectant.	Lunn et al. 2000
	TaVRT-2[c,d] *DQ022679*	Putative repressor of flowering during vernalization.	Kane et al. 2005
TaGB1 (GTP binding protein)[a] *DQ489316*	Cold acclimation induced protein ESI2-1[c] *AY666013*	Gene expression is induced during cold acclimation.	Gulick, unpublished
	Phosphatidylglycerol specific phospholipase C[c] *CK206104*	Changes in phospholipase C activity affect the expression of a large number of cold regulated genes in *A. thaliana*.	Vergnolle et al. 2005
	Phosphoinositide-specific phospholipase C[c] *DR741524*	Changes in phospholipase C activity affect the expression of a large number of cold regulated genes in *A. thaliana*.	Vergnolle et al. 2005
	Polygalacturonase[c] *CA638207*	Polygalacturonases have been connected with processes of cell expansion as wellas fruit ripening, abscission, pathogen defence and water deficit.	Hadfield and Bennett 2005; Bray 2004
TaTIL[a] *AAL75812*	Phosphatidylglycerol specific phospholipase C[c] *CK206104*	Changes in phospholipase C activity affect the expression of a large number of cold regulated genes in *A. thaliana*.	Vergnolle et al. 2005
	Phosphoinositide-specific phospholipase C[c] DR741524	Changes in phospholipase C activity affect the expression of a large number of cold regulated genes in *A. thaliana*.	Vergnolle et al. 2005
	Putative plastid ribosomal protein cl9[c] *AAM92711*		
TaVRT-1/VRN-1[a] *AY280870*	Acetylornithine transaminase[c] *BT009428*		
	Cyanate lyase[c] *CV766454*		
	Cytochrome P450-like protein[c,d] *BQ170524*	Cytochrome P450 is involved in abiotic stress response.	Narusaka et al. 2004
	Elongin[c,d] *CV765696*	Elongin complex is involved in regulation of transcription elongation by RNA polymerase II.	Gerber et al. 2005
	FK506-binding protein immunophilin[c,d] *X86903*	Immunophilins are involved in signal transduction as well as in development and stress responsiveness.	Romano et al. 2004; Romano et al. 2005
	Flowering locus T (TaFT)[e] *AY705794*	Positive regulator of flower development.	Kojima et al. 2002
	Solanum pollinated pistil-like (SPP30)[c] *AL819796*	Coded by conserved gene in evolutionarily distant organisms; predicted to have an important role in development.	Lantin et al. 1999
	Ta42G17[e] *CV765258*	Heterodimerization of MADS-box proteins is a well known phenomenon.	de Folter et al. 2005
	Ta45G05[e] *CV765903*	Heterodimerization of MADS-box proteins is a well known phenomenon.	de Folter et al. 2005
	Ta57H08[e] *CV769487*	Heterodimerization of MADS-box proteins is a well known phenomenon.	de Folter et al. 2005
	Ta73C21[e] *CV772064*	Heterodimerization of MADS-box proteins is a well known phenomenon.	de Folter et al. 2005
	Ta31K05[e] *CV762849*	Heterodimerization of MADS-box proteins is a well known phenomenon.	de Folter et al. 2005

Table 2 continued

Bait	Prey	Published co-occurrence of prey with bait, with abiotic stress, signal transduction or gene expression regulation	Ref.
	TaHd1[e] *AB094490.*	Homologous to CONSTANS, which promotes flowering of *A. thaliana* in response to long photoperiods	Nemoto et al. 2003
	TaMC44[f] Submitted to Genebank	APETALA3-like protein; Heterodimerization of MADS-box proteins is a well known phenomenon.	de Folter et al. 2005
	TaVRT-1/VRN-1[e] *AY280870*	Homodimerization of MADS box proteins is a well known phenomenon.	de Folter et al. 2005
	TaVRT-2[e] *DQ022679*	Heterodimerization of MADS-box proteins is a well known phenomenon.	de Folter et al. 2005
	VRN-2[e] *AY485975*	Zinc-finger transcription factor shown to be an important repressor of flowering; down regulated during vernalization.	Yan et al. 2004
TaVRT-2[a] *DQ022679*	AP2 domain containing protein[g] *EB714184*	Ethylene Responsive Element Binding Protein (EREBP) AP2 family member; is involved in regulation of low-temperature responsive genes.	Xue 2003
	DNA-directed RNA polymerase II 36 kDa polypeptide A[c,d] *AL820054*	Component of transcription machinery.	
	Ferrodoxin-NADP(H) oxidoreductase[c,d] *CAD30025*	Scavenger of free oxygen radicals.	Krapp et al. 1997
	Floral homeotic protein[g] *EB714175*	Homologue in Arabidopsis is known to be involved in floral organ identity and development.	Jofuku et al. 1994
	Ice recrystallization inhibition protein 1 precursor[g] *AAX81542*	Gene expression is induced during abiotic stress and jasmonic acid or ethylene treatement.	Tremblay et al. 2005
	Immunophilin[f] *X86903*	Immunophilins are involved in signal transduction as well as in development and stress responsiveness.	Romano et al. 2004; Romano et al. 2005
	LEA/RAB-related COR protein cold-responsive[g] *AAF68628*	Gene expression is inducible by cold.	Tsuda et al. 2000
	Peroxidase[c] *X56011*	Injury to plants imposed by cold exposure is associated with oxidative damage at the cellular level. Correlation between active oxygen detoxifying enzymes and chilling tolerance has been noted.	Yoshimura et al. 2004
	RING-H2 finger protein[g] *AAP80615*	RING zinc-finger proteins play important roles in the regulation of development in a variety of organisms.	Xu and Quinn Li 2003
	Ta42G17[e] *CV765258*	Heterodimerization of MADS-box proteins is a well known phenomenon.	de Folter et al. 2005
	Ta45G05[e] *CV765903*	Heterodimerization of MADS-box proteins is a well known phenomenon.	de Folter et al. 2005
	Ta57H08[e] *CV769487*	Heterodimerization of MADS-box proteins is a well known phenomenon.	de Folter et al. 2005
	Ta73C21[e] *CV772064*	Heterodimerization of MADS-box proteins is a well known phenomenon.	de Folter et al. 2005
	TaHd1[e] *AB094490.*	Homologous to CONSTANS, which promotes flowering of *A. thaliana* in responseto long photoperiods	Nemoto et al. 2003

Table 2 continued

Bait	Prey	Published co-occurrence of prey with bait, with abiotic stress, signal transduction or gene expression regulation	Ref.
	TaVRT-1/VRN-1[e] *AY280870*	Heterodimerization of MADS-box proteins is a well known phenomenon.	de Folter et al. 2005
	TaVRT-2[e] *DQ022679*	Homodimerization of MADS box proteins is a well known phenomenon.	de Folter et al. 2005
	Translation elongation factor 1 Alpha subunit[g] EB71477		
	Ubiquitin-like protein 8[c] *CV765891*	An interaction between calreticulin and ubiquitin-like nuclear protein in rice has been reported. Calreticulin is a major Ca^{2+}-sequestering protein and plays a role in cold acclimation.	Sharma et al. 2004
	VRN-2[e] *AY485975*	Zinc-finger transcription factor shown to be an important repressor of flowering; down regulated during vernalization.	Yan et al. 2004
	Wali6[g] *AAC37417*	Gene expression is inducible by aluminium.	Richards et al. 1994
α-Tubulin 3-3[b] *DQ435660*	Phosphatidylglycerol-specifc phospholipase C[c] *CK206104*	Changes in phospholipase C activity affect the expression of a large number of cold regulated genes in Arabidopsis; interaction with PLC in mammalian cells was observed.	Vergnolle et al. 2005; Popova et al. 1997
	Glycosyltransferase[c] *AJ969052*	Accumulation of zeatin *O*-glycosyltransferase in *Phaseolus vulgaris* and *Zea mays* was observed following cold stress.	Li et al. 2000

[a] Bait listed in Table 1

[b] Initially found as prey, reconfigured as bait

[c] Interaction uncovered by two-hybrid dehydration cDNA library screening

[d] Second-round confirmation of interaction was done with full-length cDNA

[e] Interaction uncovered by specific protein two-hybrid assay

[f] Interaction uncovered by BiFC

[g] Interaction uncovered by two-hybrid cold acclimated and developmental cDNA library screening

Table 3 Protein pairs confirmed by BiFC

Nt-GFP	Ct-GFP	Predominant cellular localization
TaFCA-A1	TaVRT-2	cytoplasm and nucleus
TaGB1 (GTP binding protein	Cold acclimation induced protein	cytoplasm
	PG-phospholipase C	cytoplasm
	PI-phospholipase C	cytoplasm
Pathogenesis-related protein 1	Cold acclimation induced protein	cytoplasm
	Immunophilin	cytoplasm
Receptor-like protein kinase	PG-phospholipase C	cytoplasm
	α-Tubulin	cytoplasm
Ta45G05	αTubulin	cytoplasm
αTubulin	PG-phospholipase C	cytoplasm
TaTILA	Lipid transfert protein 3	cytoplasm
TaVRT-1/ VRN-1	immunophilin	cytoplasm
	Elongin	nucleus
	Ta31K05	cytoplasm
	Ta57H08	cytoplasm
	TaVRT-1/VRN-1	cytoplasm
	TaVRT-2	nucleus
	MADS-MC44	cytoplasm
TaVRT-2	Immunophilin	cytoplasm
VRN-2	TaVRT-1/VRN-1	cytoplasm
	TaVRT-2	cytoplasm

tested. For example, reconstitution of GFP fluorescence was obtained by co-expression of TaVRT-1/VRN-1 and TaVRT-2, and was observed predominantly in the nucleus (panel A). No GFP fluorescence was detected by co-expression of either protein with the complementing non-fused GFP fragment (data not shown) or by expression of one partner with non-interacting partners (e.g. TaVRT-1/VRN-1 or TaVRT-2 with PR-1) (panel B). TaVRT-1/VRN-1 showed cytoplasmic localisation following homodimerization (panel C), which was confirmed when TaVRT-1/VRN-1 was fused to the complete GFP (panel D). The necessity of dimerization for nuclear localisation was reported for plant MADS-box proteins (McGonigle et al. 1996; Immink et al. 2002), as well as for other transcription factors (Spit et al. 1998; Chida et al. 1999). The transit from the cytoplasm to nucleus would then be part of the mechanism for the regulation of TaVRT-1/VRN-1 activity. However, BiFC experiments showed that dimerization does not always lead to nuclear localisation. TaFCA-A1 interaction with TaVRT-2 showed both nuclear and cytoplasmic localisation (Panel E), but for most cases interaction was found to be taking place in the cytoplasm (e.g. VRN-2 and TaVRT-2, panel F).

Properties of the interaction network

To evaluate the significance of the interactions, a confidence-scoring system based on Stelzl et al. (2005) was elaborated (Table 4). A quality point was given for each fulfilled criterion and the interactions were classified into categories of low (1 quality point), medium (2 quality points) and high confidence (3–4 quality points). The first criterion is that a protein–protein interaction was of higher confidence if it was able to activate several reporter genes in a reproducible manner. In our study, all the reported two-hybrid interactions activated four reporter genes, and were re-assayed for specific interactions. This is justified because several studies provide evidence that interactions that are detected with three independent reporters can be reproduced significantly more easily than interactions identified only with two reporters (Vidalain et al. 2004).The reliability of this criterion was recently confirmed by Rual et al. (2005) and Stelzl et al. (2005) who showed that interacting pairs that activated three or four reporter genes had a higher verification rate by co-purification or co-immunoprecipitation than interaction pairs that activated only two reporter genes. A second confidence criterion was confirmation by BiFC. Although selection of a given interaction pair for BiFC was not a random choice and this assay was not applied to all interaction pairs, this criterion nevertheless adds to the likelihood that the tested protein pairs interact in planta. Three- and four-protein-interaction loops were also used as a criteria for high confidence interaction scoring since these motifs are features of many biological complexes as well as pathways (Goldberg and Roth 2003; Wuchty et al. 2003; Yeger-Lotem et al. 2004). The last confidence criterion is that proteins with similar cellular function are more prone to interact with each others (Stelzl et al. 2005). Our analysis revealed that 54 interactions involved proteins with the same general process in cells (e.g. regulation of gene expression, signal transduction or response to abiotic stress), or with linked processes (e.g. regulation of gene expression with signal transduction or response to abiotic stress).

The interaction data for the library screen and specific protein interaction data is shown in Fig. 2A. On average, proteins in the network had 2.4 interaction partners. Proteins involved in three- and four-protein interaction loops are highlighted in Fig. 2B.

An interesting aspect of the interactome is the presence of a ‘‘flowering protein interaction cluster’’ represented in three- and four-protein interaction loops. This multi-protein complex contains several

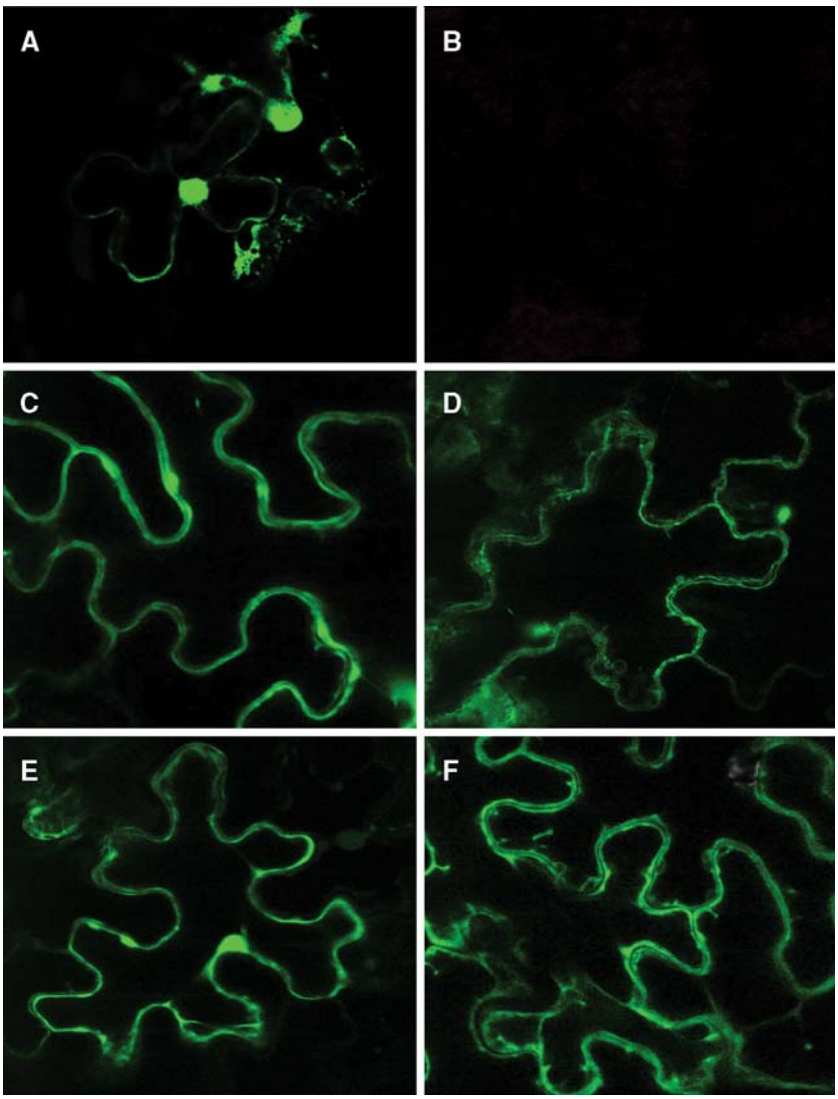

Fig. 1 Representative BiFC of selected interaction pairs uncovered by two-hybrid screening. *N.*~benthamiana leaves were agroinfiltrated with *A.*~tumefaciens supensions containing plasmids coding for: (A) ntGFP-TaVRT-1 and TaVRT-2-ctGFP; (B) ntGFP-TaVRT-1 and PR-1-ctGFP; (C) ntGFP-TaVRT-1 and TaVRT-1-ctGFP; (D) TaVRT-1-GFP; E) ntGFP-TaFCA and TaVRT-2-ctGFP; (F) ntGFP-VRN-2 and TaVRT-2-ctGFP. Reconstitution of fluorescing GFP chromophore was examined by confocal microscopy 4 days later

Table 4 Criteria for high confidence interaction scoring

Confidence criteria	Number of interactions fulfilling criteria
1 Interaction activating all four reporter genes	93
2 Interaction confirmed by BiFC	21
3 Interaction present in three- or four-protein interaction loop	37
4 Interaction where partner proteins share same or linked function	44

proteins known to be involved in flower regulation (e.g. TaVRT-1/VRN-1, TaVRT-2, VRN-2, TaAP2, TaHd and TaFT). It will thus be interesting to investigate if flowering is controlled, at least in part, by a dynamic physical interaction among transcription factors known to be inducer or repressor of flower development.

Furthermore, the interaction network shows the presence of another, overlapping, three- and four-protein interaction loops composed of signal transduction factors [e.g. two phospholipases C, a receptor-like protein kinase, a GTP-binding protein (possibly a non-canonical G protein), α-tubulin and TaTIL]. All of these proteins were shown to be involved in abiotic stress. For instance, transcript levels for the phosphoinositide-specific phospholipase C (PI-PLC) has been observed to rise rapidly in *A.*~thaliana following a cold shock (Vergnolle et al. 2005). Activation of PI-PLC by G proteins and various proteins kinases has been widely reported in mammalian studies [for a review see (Rhee 2001)]. Tubulin along with a variety of associated proteins constitute microtubules. In the case of cold-tolerant wheat, it was observed that microtubules partially depolymerised prior to the formation of cold-stable microtubules (Abdrakhamanova et al. 2003).

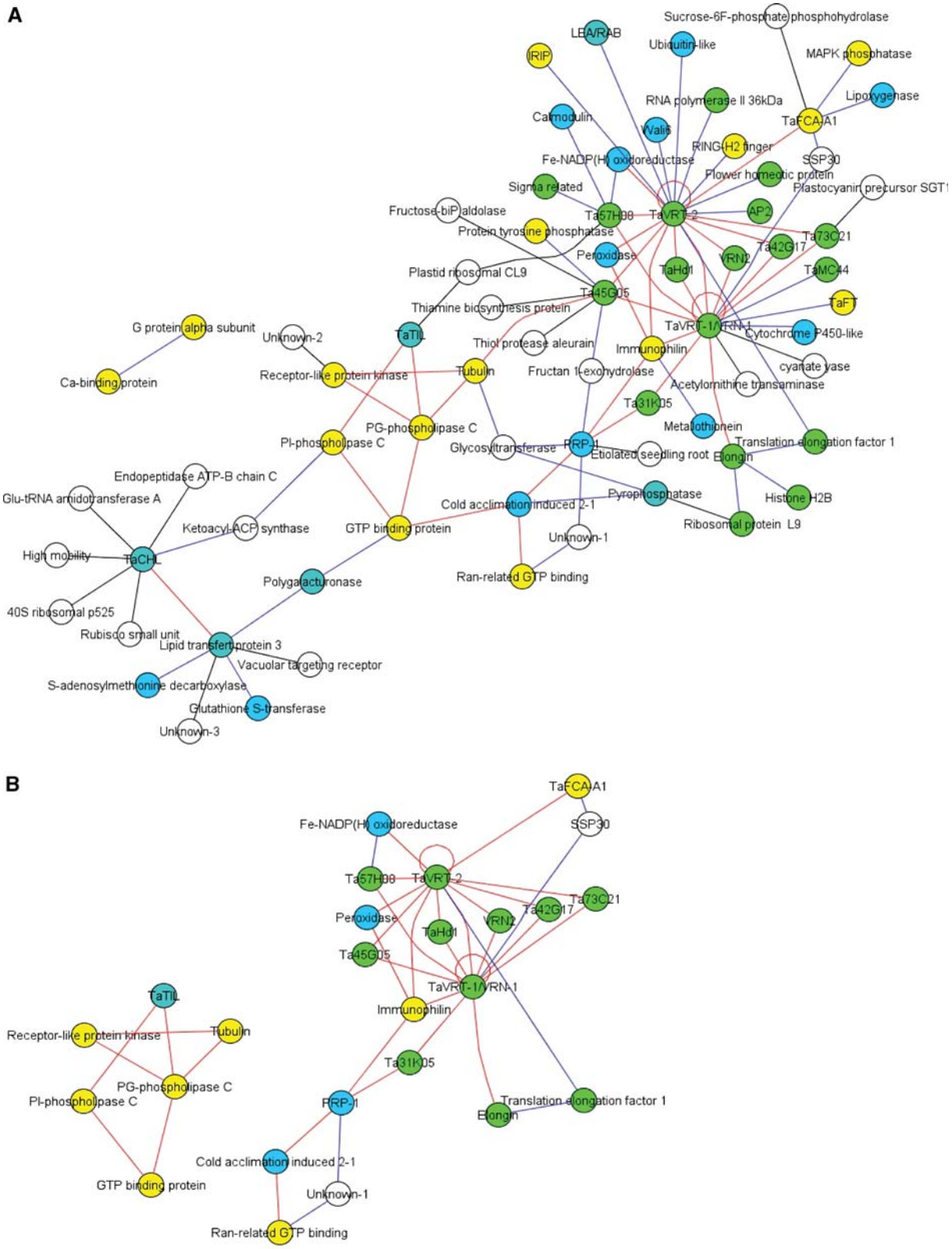

Fig. 2 (A) Network view of the abiotic stress-related interacting wheat proteins and (B) Proteins involved in three- and four-protein interaction loops. Circles depicted in green: protein involved in regulation of gene expression; yellow: protein involved in signal transduction; blue: protein involved in abiotic stress response; white: unclassified. Interactions connecting the nodes are represented by color-coded lines according to their confidence scores. Black: low confidence; Blue : medium confidence; Red: high confidence

TaTil is a lipocalin, whose transcript level has been show to rise during cold response (Frenette-Charron et al. 2002, 2005). Again, it will be interesting to investigate if signal transduction following abiotic stress is dependent on such protein–protein interactions.

This protein interaction network offers an novel insight into the cascades of protein interactions going from the cell surface to the nucleus during abiotic stress response. It provides a unique resource for further functional studies and the identification of the signalling pathways. This interaction map is currently static, and eventually the dynamics of this interactome will need to be considered to address where and when interactions take place and how they are regulated.

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