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FUNCTIONAL CHARACTERIZATION OF AN E3 UBIQUITIN LIGASE INVOLVED IN PLANT RESPONSE TO ABIOTIC STRESS

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INTRODUCTION

Plants, as sessile organisms, experience various environmental stresses including too little water (drought), too much salt (salinity), and extremes temperature. Such factors limit their development and productivity and thus prevent them from expressing the full genetic potential. Indeed, abiotic stresses are the primary cause leading to worldwide crop loss and reducing the average yield for most crops by more than 50% (Bray et al., 2000). However, plants were able to colonize many different environments indicating that they have evolved mechanisms to counteract these limiting factors.

In many plants various genes get upregulated upon exposition to stress resulting in altered metabolic functions, which can mitigate the effects of stress and lead to plant adaptation. Plant responds to the stress as individual cell and synergistically as a whole organism.

The molecular mechanisms underlying the plant tolerance response to stress involve three main steps: firstly the stress signal is perceived by the receptors present on the surface of cell membrane; secondly this signal is transduced to the transcription factors present into the nucleus. Upon receiving the signal, transcription factors activate the expression of target stress inducible genes which ultimately lead to plant adaptation and help the plant to survive and surpass the unfavorable conditions (Trewavas et al., 1997; McCarty et al., 2000; Gilroy et al., 2001).

Since the beginning of studies unraveling stress tolerance in plants, the main research focus have been the identification of genes whose expression profile changes upon perceiving the stress and the mechanisms of stress signal perception (receptors and secondary messengers). Indeed, in the last decade, the availability of high-throughput system for large transcriptome analyses like

microarrays allowed the identification of hundreds of stress inducible genes in different plant species. Notwithstanding, with the final goal to modify plant sensitivity to stress, many efforts of biochemical studies have been dedicated to the sensing systems and to dissect the intricate signal transduction pathways responsible for the activation of signalling cascades leading to tolerance response. Transcription factors are them selves controlled by many regulatory proteins involved in the signal transduction, protein modifiers responsible of post translational modifications (like methylation, sumoylation, ubiquitination, phosphorylation) as well as adaptors and scaffolds mediating protein-protein interactions and the formation of active protein complexes. Indeed some stress-related transcription factors have been found activated following a post-translational modification in response to stress signals. Experimental evidence indicates that further levels of changes beyond transcriptional ones are activated following exposure of plants to stress and that they are fundamental in the regulation of plant response to stress. Therefore during recent years the attention of the researchers has expanded beyond changes in transcriptional profile during plant stress response to include post transcriptional and post translational events.

Various steps of RNA processing affect quantitatively and qualitatively the mRNA population. Alternative splicing, which concerns up to two thirds of the genes, has important consequences on the availability of different kinds of transcripts, and ultimately of proteins (Ner-Gaon et al., 2007). RNA-mediated silencing is also emerging as an alternative mechanism to control the amount of specific transcripts by their degradation (Sunkar et al., 2007). Subsequent steps of RNA processing, like the mRNAs transport through the nuclear envelope and the association of mRNAs to ribosomes, are other important check points of mRNA availability for protein synthesis (Xu et al., 2008; Branco-Price et al., 2005).

After the translation, a plethora of molecules can constitutively or transiently interact with proteins modifying their activity, sub-cellular localization and half-life (Downes et al., 2005). Phosphorylation is one of the best known post-translational protein modifications affecting conformation, activity, localization and stability of target proteins. It has a role in many biological processes, as phosphorylation cascades commonly translate extracellular stimuli into the activation of specific responses (Boudsocq et al., 2005). Among the polypeptides, ubiquitin and SUMO conjugations are emerging as major post-translational regulatory processes in all eukaryotes (Stone et al., 2007; Miura et al., 2007).

From the most recent studies, these post transcriptional/translational mechanisms as well as their interactions, are raising as key regulatory systems of the transcriptional changes controlling the plant response to stresses. They finely modulate the amount and activity of pre-existing transcripts and proteins, respectively, with an ultimate effect on proteome and metabolome complexity (Oksman-Caldentey et al., 2005; Reinders et al., 2007). The molecular response underlying plant tolerance must be promptly as well as finely modulated depending on stress intensity and duration. To achieve this fine regulation, the activity of proteins are regulated by different types of post-translational modifications. The network of such mechanisms is expected to effectively target transcription factors and other regulatory components of the stress signaling, resulting in either activation or repression of their activities. This ensures temporally and spatially appropriate patterns of downstream gene expression and ultimately the shaping of transcriptome and proteome of stress-exposed plants to switch on adaptive response. Although stress-responsive genes still represent an important aspect of stress adaptation, the simple observation of the transcriptome provides only a rough and largely incomplete picture of the response to stress. The characterization of posttranscriptional and post-translational regulatory systems is crucial for the deeper understanding of the

molecular mechanisms governing plant adaptation to environment as well as for a practical purpose of plant improvement for stress tolerance.

UBIQUITINATION AND 26S PROTEASOME: AN OVERVIEW

Discovered 30 years ago, the 76 amino acid protein ubiquitin was richly deserved the nickname: especially in plants kingdom during the past decade ubiquitination has been connected to almost all aspect of plant biology. Furthermore is now clear that ubiquitin is only one of the polypetides that have a common ubiquitin fold (Downes et al., 2007; Kerscher et al., 2006). Mainly ubiquitin-like polypeptides are attached in a covalent manner to a target protein in order to promote protein-protein interaction. Collectively, these polypeptides act as portable recognition modules that promote protein–protein interactions after their conjugation to various intracellular constituents.

In the case of ubiquitin, targets are other proteins whose fate depend on number of ubiquitin molecules and location of the polyubiquitin chain (Figure 1; Vierstra 2009).

Ubiquitin itself has different lysine residues which position is very well conserved among all eukaryotic organism: Lys-11, Lys-29, Lys-48 and Lys-63. All these Lysine residues can form ubiquitin-ubiquitin isopeptide linkage *in vivo* and the type of linkage strongly influences the consequence of ubiquitination and the fate of target proteins. Furthermore, a target protein can be modified at one or more Lysine residues by a single ubiquitin molecule (monoubiquitination), by Lysine-linked chains of ubiquitin (polyubiquitination) or by a combination of both (Bach et al., 2003).

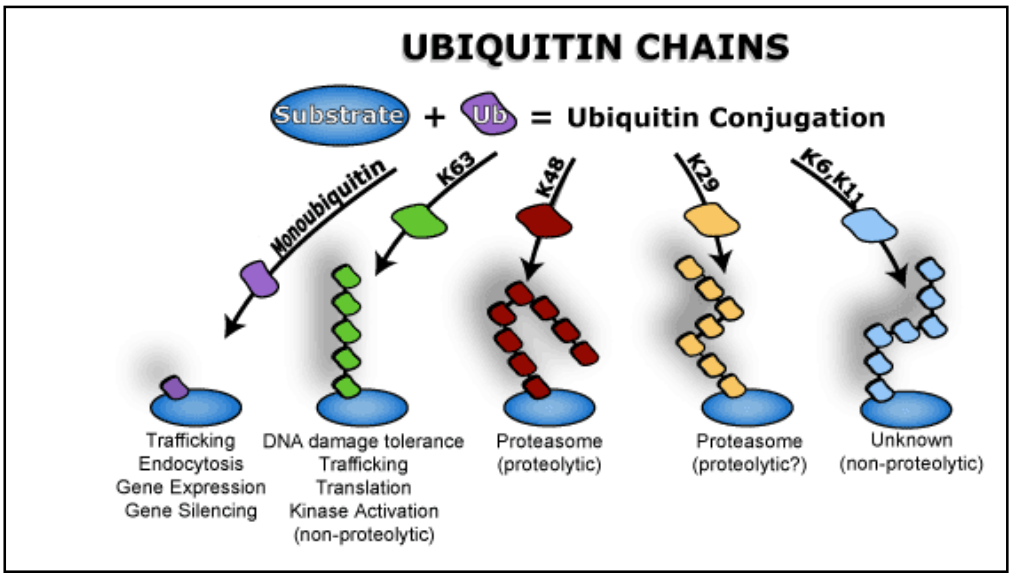


Figure 1: fate of ubiquitinated proteins. The fate of ubiquitinated proteins strongly depends from the length and the type of ubiquitin chain.

Lys-48 residue can be further conjugated by another ubiquitin moiety in a processive manner to form a polyubiquitin chain of at least four ubiquitins. The most famous fate of proteins labeled with this polyubiquitin chain is the proteasome 26S-mediated degradation, which is also the best characterized function of ubiquitination. Besides the elimination of aberrant or truncated proteins for cellular housekeeping, this pathway regulates the amount of active proteins, which depends on the synthesis/degradation ratio.

The 26S proteasome is a 2-MDa ATP-dependent proteolytic complex (Smalle and Vierstra, 2004). It is constituted by 31 principal subunits that form two subcomplexes, the 20S core protease (CP) and the 19S regulatory particle (RP) (Figure 2). The CP is a cylindrical stack with ATP- and Ub- independent protease activity, formed by the assembly of four heptameric rings. The two peripheral rings are composed of seven related α subunit while the two central rings are composed of seven related β subunit in a $\alpha_{1-7}/\beta_{1-7}/\ \beta_{1-7}/\alpha_{1-7}$ configuration (Smalle and Vierstra, 2004). The large central ring of the CP

houses the protease active sites provided by β_1, β_2 and β_5 subunits. They belong to the NTn hydrolase family that uses an N-terminal threonine as the active-site nucleophile; this residue is exposed following cleavage of a propeptide.

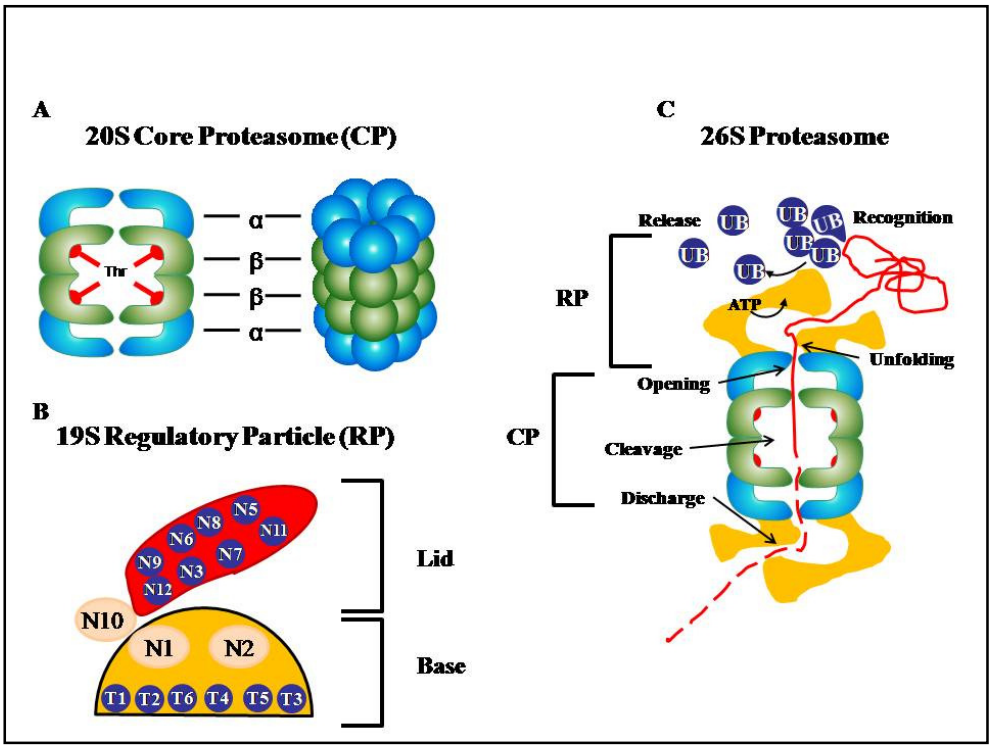


Figure 2: Organization and structure of the 26S proteasome (modifeid from Smalle and Vierstra, 2004). (A) Organization of the 20S core protease (CP) based on the crystal structure of the yeast particle. The positions of the active-site threonines are shown. (B) Predicted organization of the 19S regulatory particle (RP) based on its subunit interaction map with the Lid and Base shown in red and yellow, respectively. The RPAAA-ATPase (RPT) subunits are shown in blue. The RP non-ATPase subunits are shown in orange. (c) Diagram of the 26S proteasome combined with the predicted activities of the complex during the degradation of ubiquitinated proteins.

The β1, β2, and β5 subunits generate peptidylglutamyl peptide-hydrolyzing, trypsin-like, and chymotrypsin-like activities, respectively, thus conferring to the CP the capacity to cleave most, if not all, peptide bonds. Like the yeast and mammal counterparts, the active sites of plant CP are very sensitive to the 26S proteasome inhibitors, MG115, MG132, lactacystin and epoxomycin. The α subunits-composed rings restrict the access to the circular β-composed channel

such that only unfolded proteins may enter. The flexible N terminal extension of α subunits regulate substrates entry and products exit allowing to the CP to spatially separate proteolysis from the cellular milieu and restricts degradation to only those polypeptides that are deliberately unfolded and imported (Smalle and Vierstra, 2004).

The RP associates with one or both ends of the CP and confers both ATP dependence and a specificity for Lys48-linked polyubiquitin chains to the particle. The RP is composed of 17 core subunits that can be further divided into the Lid and Base subcomplexes (Figure 3). The Base takes place directly over the α rings channel and contains a ring of six related AAA-ATPase (RPT1-6) and three non-ATPase subunits (RPN1, 2 and 10). The Lid contains the remaining non-ATPase subunits (RPN3,5-9 and 11-12). The role of RP is to assist in recognizing and unfolding appropriate substrates, removing the covalently bound Ubiquitins, opening the *α*-ring gate, and then directing the unfolded polypeptides into the lumen of the CP for breakdown (Smalle and Vierstra, 2004).

DIFFERENT FATES OF UBIQUITINATED PROTEIN

Lys-48 mediated polyubiquitination not always targets protein to 26S proteasome mediated degradation (Li et al., 2008), indeed recent studies also reveal some novel non-proteolytic functions for Lys48-linked polyubiquitin chains. One example is the regulation of *Saccharomyces cerevisiae* Met4 transcription factor. Met4 activates the expression of genes in the methionine biosynthetic pathway and its activity is tightly regulated by the level of the end product (methionine in this case) like many biosynthetic regulators. When the yeast cells growth on a medium containing methionine Met4 undergoes

ubiquitination losing its transcriptional activity. Interestingly although Met4 is conjugated with Lys48-linked polyubiquitin chain it is not degraded via 26S proteasome. It seems possible due to the presence on Met4 of a domain able to interact with ubiquitin chain maintaining the length of the ubiquitin chain below the threshold required for proteasome recognition (Li et al, 2008).

Mono- or poly-ubiquitination via Lys-63 residue is likely to mediate non-proteolytic events. For example, Lys-63 (K63)-linked polyubiquitin chains have been shown to mediate human protein kinase activation, DNA repair and vesicle trafficking. Monoubiquitination has also shown to be involved in nuclear targeting of human proteins interacting with DNA repair enzymes (Sun et al., 2004). How ubiquitination can regulate the activity of target proteins is still unclear: ubiquitination could change the conformation of target proteins, otherwise a monoubiquitin moiety or a distinct polyubiquitin chain could serve as specific protein-protein interaction domain to recruit proteins carrying various ubiquitin-binding domains.

Less is known about ubiquitination *via* other residues although several recent studies have begun to explore the physiological relevance of them. Recently was demonstrated that AIP4, a HECT domain E3 ligase, can assemble Lys29-linked ubiquitin chain on the Notch signaling modulator DTX to target it for lysosomal degradation in *Drosophila* (Chastagner et al., 2006). Another study, in human field, showed that two AMPK-related kinases may be modified with Lys29 and/or Lys33-linked ubiquitin chains in cells and such modification appears to regulate the enzymatic activity of these kinases (Al-Hakim et al., 2008).

THE ENZYMATIC CASCADE THAT DIRECTS UBIQUITINATION

The ubiquitin attachment to target proteins is mediated by a three-steps enzymatic cascade (Figure 3). In the first step a thioester bond between ubiquitin and the ubiquitin-activating enzyme E1, is formed in an ATP-dependent reaction. Subsequently, ubiquitin is transferred to the ubiquitin-conjugating enzyme E2. Then, the transfer of ubiquitin to the target protein occurs in presence of the ubiquitin ligase enzyme E3, which specifically recognizes the target proteins. An isopeptide bond is formed between the carboxyl terminus of ubiquitin and the ε-amino group of a lysine residue on the target protein. The efficient polyubiquitination is facilitated by multiubiquitin chain assembly factors (E4) which transfer additional ubiquitin moieties longer than di- or tri-ubiquitin (Koegl et al., 1999).

In a recent work (Du et al., 2009) a database of higher plants' UPS (Ubiquitin Proteasome System) comprising *Arabidopsis*, rice, poplar, soybean, grape, medicago and maize, named plantsUPS, has been developed underling the increasing interest of researchers in studying the role of ubiquitination during plant life cycle (http://bioinformatics.cau.edu.cn/plantsUPS/).

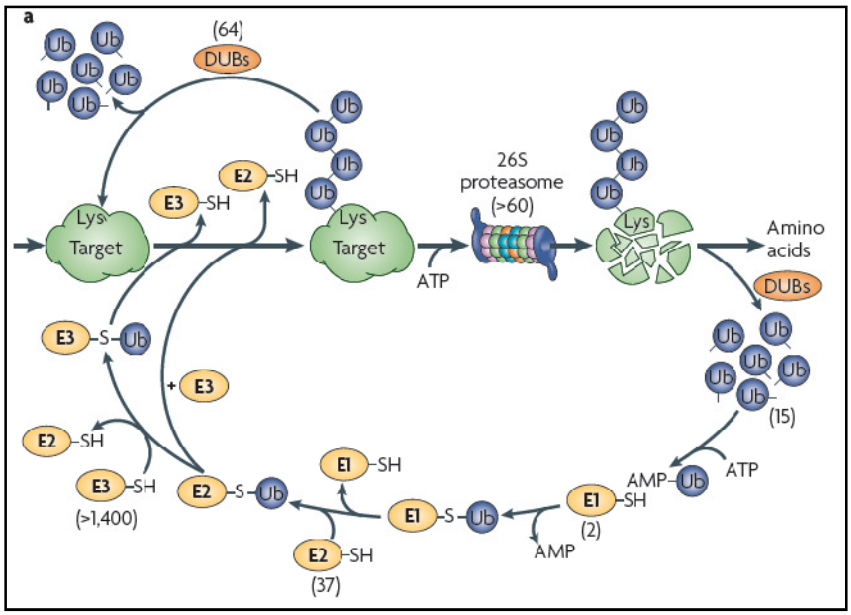


Figure 3: (modified from Viestra 2009), graphic representation of enzymatic cascade of ubiquitin pathway; the numbers in parentheses indicate the predicted number of genes that encode each UPS (Ubiquitin Proteasome System) component in Arabidopsis thaliana.

THE E3 UBIQUITIN LIGASE GENE FAMILY IN PLANTS

From: ***The E3 Ubiquitin Ligase Gene Family in Plants: Regulation by Degradation***

(Mazzucotelli, Belloni, Marone, De Leonardis, Guerra, Di Fonzo, Cattivelli, Mastrangelo, 2006)

The most crucial function within the ubiquitination pathway is the specific recognition of target proteins. This function is accomplished by the E3 enzymes, each E3 ubiquitin ligase targets a particular protein. Many genomic studies have been carried out to identify all potential E3 ubiquitin ligase sequences in plants, particularly in species whose genome has been completely sequenced as *Arabidopsis* and rice. More than one thousand E3 ubiquitin ligase members have been predicted in *Arabidopsis* (Table 1). The huge size of the E3 ubiquitin ligase gene family and the wide range of *stimuli* in which they are involved (Fig. 4) agrees with its critical role to give specificity to the system.

E3 sub group	Abbreviation	Number of genes	References
Homology to E6-AP C-Terminus	HECT	7	Downes et al., 2003
Really Interesting New Gene	RING	499	Kosarev et al., 2002 Mladek et al., 2003 Stone et al., 2005 Serrano et al., 2006
Plant U-Box	PUB	49	Azevedo et al., 2001 Luo et al., 2006 Mudgil et al., 2004
CULLIN	CUL	11	Shen et al., 2002 Gingerich et al., 2005
Cyclin F proteins	F-Box	724	Risseeuw et al., 2003 Gagne et al., 2002 Kuroda et al., 2002 Wang et al., 2004
Arabidopsis Skp1-related	ASK	21	Farras et al., 2001 Marroccoet al., 2003 Zhao et al., 2003
Bric a Brac, Tramtrack and Broad complex	BTB	81	Gingerich et al., 2005 Dieterle et al., 2005
CULLIN4-Damaged DNA-Binding Protein	CUL4-DDB	5	Yi et al., 2005 Koga et al., 2005
Anaphase Promoting Complex	APC	18	Capron et al., 2003
	Total	**1415**	

Table 1: (from Mazzuctelli et al., 2006); genomic organization of E3 ubiquitin ligases gene family.

E3 ubiquitin ligases are classified into two groups depending on the presence of a HECT (Homology to E6-AP C-Terminus–Figure 5a) or a RING (Really Interesting New Gene)/U-box domain. RING proteins can act as single components containing both the active site and the binding pocket for the E2-ubiquitin intermediate (Figure 5b), or as components of multisubunit complexes which in plants include SCF (SKP1-CULLIN-F-box), CUL3 (CULLIN 3)-

BTB/POZ (Bric a brac, Tramtrack and Broad complex/Pox virus and Zinc finger), CUL4-DDB1 (UV-Damaged DNABinding Protein 1) and APC (Anaphase Promoting Complex) complexes, as shown in Figure (5c, d, e and f).

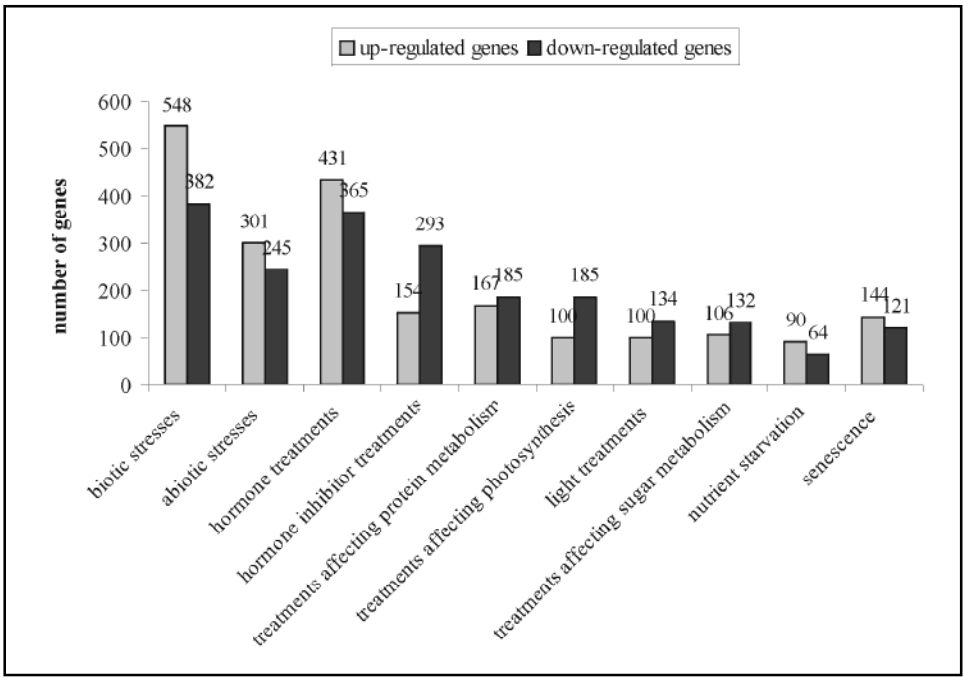


Figure 4: Changes in genes expression of E3 ubiquitin ligase genes following different conditions. (from Mazzucotelli et al., 2006) Up-regulated genes are reported in grey, down-regulated genes in black. Number of up- and down-regulated genes is indicated for each condition. The conditions considered are: biotic stresses; abiotic stresses; hormone inhibitors, treatments affecting protein metabolism, treatments affecting photosynthesis, light treatments, treatments affecting sugar metabolism, nutrient starvation and senescence.

While RING/U-box E3s generally act as molecular adapters between E2 and target proteins, the HECT E3s form a covalent bond with ubiquitin before transferring it to the protein substrate, using a conserved Cys of the HECT domain to form a ubiquitin-E3 thiole-ester intermediate (Pickart et al., 2001).

The multi-subunit E3 ligases are CULLIN and RING finger-based protein complexes. These enzymes are constituted by two functional modules. The catalytic module is composed by a RING-finger subunit (RBX1- RING Box protein 1-or APC11) interacting with the E2 enzyme. The second module

(adapter) specifically recognizes target substrates for ubiquitination. The same catalytic module can be associated to many different adapters, therefore many different E3 complexes are formed, which in turn catalyze the ubiquitination of different substrates. The two modules are brought together by a CULLIN (or CULLIN-like, APC2) protein which acts as a molecular scaffold and also defines the E3 class.

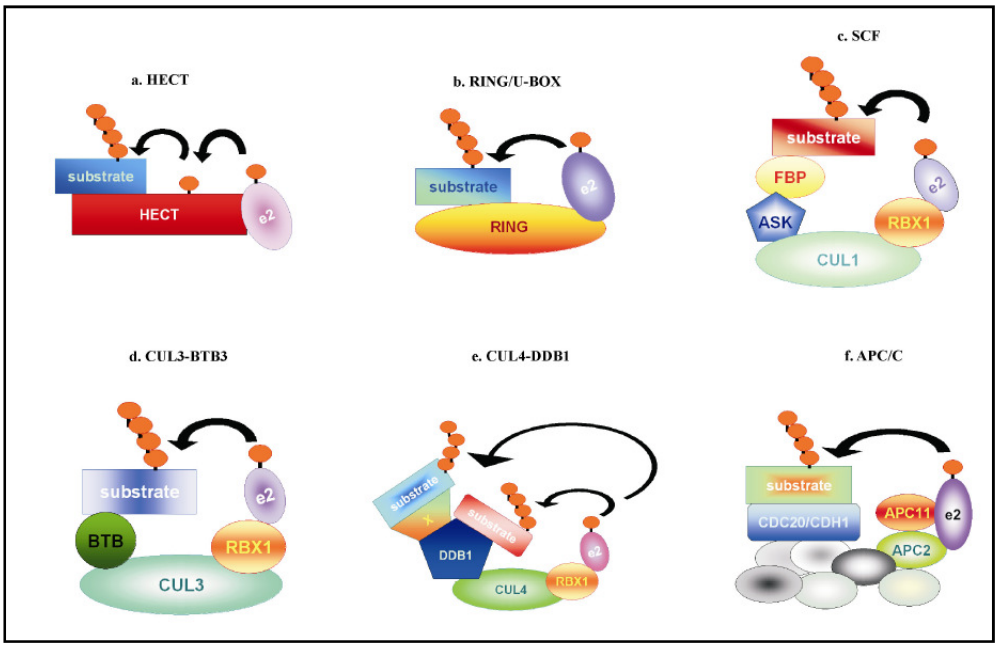


Figure 5: (from Mazzucotelli et al., 2006); organization of E3 ubiquitin ligases. CULLIN and CULLIN-like proteins are in green and RING proteins are in red/orange; small linked circles represent polyubiquitin chains. (a) and (b): E3 single components. (c)-(f): E3 multi subunit complexes.

The E3 SCF complexes contain four core components: SKP1 (S-phase Kinase associated Protein 1, named ASK1, for *Arabidopsis* Skp1- related in plants), CUL1, a F-box protein and RBX1. SCF structure has been resolved (Zheng et al., 2002) in yeast and mammals. RBX1 catalyses the synthesis of polyubiquitin chains and, together with CUL1, forms a catalytic core complex recruiting a cognate E2. The CULLIN subunit acts as a large scaffold protein that ensures optimal presentation of the substrate to the E2 enzyme. It binds both RBX1 and the linker protein SKP1. The SKP1 protein serves as an adapter

between CUL1 and the variable F-box protein, which binds the substrate (Bai et al., 1996; Skowyra et al., 1997; Schulman et al., 2000).

The second family of the multisubunit E3 complexes is defined by the association among the highly conserved CULLIN family member, CUL3, a BTB/POZ domain protein, and the RBX1 protein (Pintard et al., 2004). In the current animal model for CUL3-BTB E3 ligases, BTB proteins function as substrate-specific adapters, which bind CUL3 through its BTB-domain and interact with the substrate through an associated protein-protein interaction domain (Geyer et al., 2003). RBX1 binds the E2 enzyme probably leading to its allosteric activation (Furukawa et al 2002). The BTB/POZ domain appears to assume a three-dimensional structure similar to the CUL1 interaction domain of the SKP1 adapter proteins (Schulman et al., 2000), therefore it has been suggested that BTB proteins function as SKP1/F-box hybrid proteins that deliver the targets to CUL3.

CUL4 protein is a core component of a new class of E3 ubiquitin ligases that regulate replication and transcription in mammals. These E3 ubiquitin ligases also contain the DDB1 and RBX1 proteins. CUL4-DDB1 may recruit substrates directly, or through additional factors, such as the complex formed by De-Etiolated-1 (DET1) and Constitutively Photomorphogenic-1 (COP1) proteins (McCall et al., 2005).

The APC is a multiprotein complex, constituted by eleven subunits conserved in all eukaryotes, involved in the regulation of the cell cycle progression by degrading cyclins. Two APC proteins, APC2, a distant member of the CULLIN family, and the RING-finger protein APC11 form the minimal ubiquitin ligase module of the APC. The two proteins interact with each other and with E2 ubiquitin conjugating enzymes, and together are able to catalyze ubiquitination of proteins in vitro, although without substrate specificity (Gmachl et al., 2000). Stage-specific activation of APC as well as selection and binding of the

substrates depend on a WD40 protein able to interact with the substrate. Two types of WD40 proteins have been identified, Cdc20 (Cell division cycle) and Cdh1 (Cadherin 1), known as Ccs52 (Cell cycle switch 52) in plants, and two different complexes can be formed depending on which WD40 protein interacts with the other APC subunits. In human APC, the interaction between the WD40 proteins and the APC core is mediated by two additional subunits (APC3 and APC7) (Vodermaier, 2003).

RING-FINGER E3 UBIQUITIN LIGASES

RING containing proteins represent, together with F-box proteins, the most abundant E3 ubiquitin ligase gene families. The RING finger motif was first identified in the protein product of the human gene RING1-Really Interesting New Gene 1 (Freemonte et al., 1991).

The RING domain contains four pairs of zinc ligands binding coordinately two zinc ions (Barlow et al., 1994; Borden et al., 2000). The zinc ligands are formed by cysteine and histidine residues placed at proper distance in a typical cross-brace structure. The two main RING domains, C3HC4 and C3H2C3, contain a conserved histidine at metal ligand position 4, but differ for the presence of either a cysteine or cistidine residue, respectively, at metal ligand position 5. The RING domains were shown to be essential for E3 ubiquitin ligase activity of human RING-containing proteins (Lorick et al., 1999).

Only a limited number of RING proteins with a predicted or known biological function have been characterized in plants. The complete sequence of the *Arabidopsis* and rice genomes gave new chances for large gene families characterization in plants. Indeed, systematic searches for RING-finger domain containing proteins yielded about 500 distinct sequences in *Arabidopsis*, which

should represent an exhaustive list of RING genes in this species (Kosarev et al., 2002; Mladek et al., 2003; Stone et al., 2005; Serrano et al., 2006).

Kosarev et al. (2002) identified 365 genes grouped into six clusters based on sequence similarity and RING domain features. A more extended search, performed by Stone et al. (2005) led to the identification of 469 genes, characterized by 3 RING-type (RING-H2, RING-Hca and RINGHCb) and five modified RING-type domains (RING-C2, RING-v, RING-D, RING-S/T and RINGG) based on the nature and on the distance between the metal ligand residues. The majority of analyzed proteins contained only the RING-finger domain (about 150 sequences) or a RING motif associated with one or more transmembrane domains (about 120 sequences). In the remaining sequences, the RING motif was found associated to a number of known domains which may interact with the target protein, or act as regulatory components. Many RING-associated domains, such as coiled-coil or zinc finger Cx2Cx5Cx2C motifs and Ankyrin repeats, are potentially involved in the protein-protein interaction mechanisms. Furthermore, some domains are supposed to interact with specific class of proteins, i.e. the WD40 repeats show specificity for ser/thr phosphoproteins (Orlicky et al., 2003).

A well characterized RING finger E3 ubiquitin ligase is COP1, which functions as central switch in light control of *Arabidopsis* seedling development. COP1 protein contains three structural domains: a RING finger, followed by a coiled-coil domain and seven WD40 repeats at the C-terminus.

A large set of proteins interacting with COP1 have been identified. In particular, it has been proven that the RING domain mediates the interaction with COP10, a protein functioning as E2 ubiquitin conjugating enzyme, and CIP8 (COP1 Interacting Protein 8), another E3 ligase also involved, together with COP1, in the ubiquitination of the transcription factor HY5 (long hypocotyls phenotype 5). The coiled coil domain allows the dimerisation of

COP1 and its interaction with a number of polypeptides, such as SPA (Supressor of phyA) proteins, involved in the modulation of COP1 activity. Finally, the C-terminal WD40 repeats are involved in the recognition of the ubiquitination targets: the transcription factors HY5, HYH (HY5-like protein H) and HFR1 (long Hypocotyl in Far Red 1) and the photoreceptors cry2 and phyA (Yi et al., 2005).

In some cases the RING domain was found associated to putative nucleic acid binding motifs such as C2H2 and C3H1 (DNA-binding zinc finger domains), or KH (K Homology) and RRM (RNA Recognition Motif). Notably, one RING finger protein (At3g54460) is predicted to contain an F-box domain (Stone et al., 2005).

ARIADNE proteins are characterized by the IBR (In Between Ring) domain, a motif with the pattern C6HC, usually located between two RING domains. The *Arabidopsis* ARIADNE family is formed by 16 genes (Mladek et al., 2003); twelve of them clustered together by Stone et al. (2005).

ATL gene family members were originally isolated as genes rapidly induced in response to elicitors (Salinas-Mondragon et al., 1999). They contain a RING-H2 type domain with a particular signature: a highly conserved proline spaced out a residue upstream from the third zinc ligand, and a highly conserved tryptophan spaced out three residues downstream from the sixth zinc ligand. The analysis of Serrano et al. (2006) led to the identification of 80 ATL proteins in *Arabidopsis*, most of them clustered together according to Kosarev et al. (2002) and to Stone et al. (2005).

Despite extensive studies on the RING protein family have been performed only in *Arabidopsis*, some evidences exist that the organization of this family can vary in different plant species. The modified RING domain RING-D appears to be characteristic of *Arabidopsis*(or dicots) species, since a similarity

search carried out in rice genome failed to find proteins with the same domain (Stone et al., 2005).

The *Arabidopsis* RING gene collection also comprises three genes coding for proteins acting as subunits of large complexes (AtRbx1;1 and AtRbx1;2) whose protein products participate in assembling of SCF, CUL3-BTB and CUL4-DDB complexes (Lechner et al., 2002), and the APC11 gene, involved in the formation of the APC complex (Capron et al., 2003a; Capron et al., 2003b).

UBIQUITIN-MEDIATED DEGRADATION: A RECURRENT THEME IN PLANT LIFE CYCLE

Despite the huge knowledge obtained on large E3 ligase family in plants from a genomic point of view, wide information are still lacking about individuation of target proteins, that remains the most intriguing, and poorly understood aspect of the ubiquitination pathway, which, by specifically affecting many components of cellular regulation, can participate to the fine tuning of cellular response to the variable life conditions. The current knowledge on the role of UPS in plant cell is based above all on genetic studies of mutants in genes encoding components of the ubiquitination system, in particular E3 ligases. The general outcome of these studies is that most aspects of plant life cycle are somehow regulated by ubiquitin-mediated degradation of key proteins. This feature gets critical when target proteins are short-lived regulatory components, like enzymes directing rate-limiting steps, signaling receptors and transcriptional factors (Smalle et al., 2004).

Notably, an effect frequently observed in E3 ligase *Arabidopsis* insertional mutants is the arrest of embryo development. For four T-DNA insertional lines for ATL-RING genes only hemizygous lines were recovered, suggesting that the

mutated genes could be essential for plant viability (Serrano et al., 2006). The embryo lethality associated to gene disruption was also described for other RING E3 ubiquitin ligases, as the *Arabidopsis* RIE1 (Xu et al., 2003) and PEX12. The analysis of RNA interference plants with partial reduction of the PEX12 transcript, exhibiting impaired peroxisome biogenesis and function, addressed the PEX12 function to the regulation of the peroxisome development (Fan et al., 2005). Since RBX1, ASK and CUL proteins participate to form several E3 complexes, they could be involved in the control of many different processes, therefore, in absence of functional redundancy, many loss of function mutants may lead to a lethal phenotype. Indeed, *Arabidopsis* mutants containing T-DNA insertions in the CUL1 gene displayed an arrest in early embryogenesis, before the first cell division of both embryo and endosperm cells (Shen et al., 2002). Consistently, both the transcript and the product of the CUL1 gene were found to accumulate in embryos. The CUL1 protein was localized mainly in the nucleus, weakly present in the cytoplasm during interphase and co-localized with the mitotic spindle in metaphase.

CUL3A and CUL3B appear functional redundant, since cul3a and cul3b mutants are viable and, consistently, they have largely overlapping expression patterns. However, the disruption of both the CUL3A and CUL3B genes affected both embryo pattern formation and endosperm development, causing embryo lethality (Gingerich et al., 2005, Figueroa et al., 2007, Thomann et al., 2005). Although arrest at the heart stage was predominant, block of embryogenesis occurring at multiple stages of embryo development indicated a general growth inhibition of the embryo.

CUL4-DDB1 complex also appears essential, being the ddb1b mutation embryo lethal, although *Arabidopsis* contains two highly related DDB1 proteins (Schroeder et al., 2002). According to RBX1 involvement in the formation of SCF, CUL3-BTB and CUL4-DDB1 complexes, severe phenotypes were observed for *Arabidopsis* RBX1 dsRNA lines, consistently with the suppression

level (Lechner et al., 2002), including the block of seedling development, death of young seedlings, or severe dwarf phenotypes. A recent work (Bernhardt et al., 2006) has proven that *Arabidopsis* CUL4 participates in important processes such as the cell cycle, light-dependent growth control, modulation of chromosomal structure and DNA repair. *Cul4* mutants are also severely affected in different aspects of the development: a reduced level of *CUL4* expression leads to a reduced number of lateral roots, to abnormal vascular tissue and stomatal development, and to weakly altered response to photomorphogenic stimuli. These findings strongly supported the essential role of E3 complexes in many plant biological processes activated during plant embryogenesis and post-embryonic development.

Deregulation of RBX1 protein level in dsRNA lines and mutations in *ASK1* gene led also to phenotypes with reduced auxin response and decreased jasmonate response, similar to those observed in the axr1, tir1, and coi1 mutants, substantiating the role of RBX1 and ASK1 as components the SCFTir1 and SCFCoi1 complexes, known to be involved in auxin and jasmonate signaling respectively (Gray et al., 1999, Becker set al., 2006, Ren et al., 2005).

Ask1 mutants and RNAi lines showed also severe flower defects (Zhao et al., 2003), in accordance to previous evidences of association between ASK1 and the F-box protein UFO (Unusual Floral Organ), involved in flower development and male sterility (Zhao et al., 2001, Wang et al., 2004, Wang et al., 2006). Although a role of ASK1 in GA-signal transduction is not yet demonstrated, the SCF-complex SLY (SLEEPY) regulates GA response (McGinni et al., 2003) and the RNAi line showed also features similar to those found in GA-deficient or insensitive mutants (Zhao et al., 2003). ASK1 was found to interact also with EID1, a F-box involved in phytocrome-A specific light signaling (Marrocco et al., 2006).

The F-box proteins EBF1/2 (EIN3-Binding F-box) (Potuschak et al., 2003) known to belong to the ethylene signaling pathway, ORE9 (ORESARA9 - "long living"), are involved in the regulation of plant leaf senescence (Woo et al., 2001), whereas the F-box protein LKP2 (LOV KELCH Protein 2) and ZTL (ZEITLUPE) are involved in the regulation of circadian timings (Yasuhara et al., 2004, Han et al., 2004). The proteomes of the *Arabidopsis* wild type and ask1 mutant flower buds have been compared evidencing ten proteins involved in photomorphogenesis, circadian oscillation, post-translation process, stress-responses and cell expansion or elongation, all processes being misregulated in the mutant (Wang et al., 2006). Although the implication of ASK1 in various cell processes, ask1 mutants are viable. In a similar manner, although the ASK2 protein was found to be associated with several F-box proteins, such as TIR1 (Transport-Inhibitor Response 1) (Gray et al., 1999), COI1 (Coronatine-insensitive) (Xu et al., 2002), UFO (Samach et al., 1999), and EID1 (Dieterle et al., 2001), ask2 mutant is morphologically similar to wild type. Therefore partially functional redundancy has been evocated to explain the viability of the two mutants. Indeed, the ask1-ask2 double mutation affects cell division and cell expansion/elongation, resulting in delayed embryogenesis and seedlings lethality, and demonstrating a vital role for ASK1 and ASK2 in embryogenesis and postembryonic development (Liu et al., 2004).

Functional analyses have been accomplished for some BTB proteins linking individual BTB proteins to several processes in plants. The best characterised BTB protein is ETO1 (Ethylene- Overproducer 1), a protein involved in the control of ethylene biosynthesis. As reported above, it is able to interact with CUL3A, likely forming a BTBETO1. Moreover it was found to target for degradation of the ACC synthase ACS5, the rate limiting enzyme of the ethylene biosynthesis and the first reported substrate for a plant CUL3-based E3 (Wang et al., 2004).

For other *Arabidopsis* BTB proteins, possible functions have been inferred biochemically, as the transcriptional regulation activity of some members of BTB family, characterised by a calmodulin-binding domain, in response to H2O2 and salicylic acid treatments (Du et al., 2004). FIP2 (Rab11- Family of Interacting Protein 2) protein was found to interact with *Arabidopsis* Formin Homology 1, AFH1 (Banno et al., 2000), a protein required for polar pollen tube extension (Cheung et al., 2004). The involvement of E3 ubiquitin ligases in signaling of hormones as ABA, jasmonic and salicilic acids indicates a strong functional role of ubiquitination pathway in plant response to environmental clues. The most studied system is represented by COP1/CIP8 and their role in regulation of photomorphogenesis (Yi et al., 2005, Hoecher et al., 2005, Roig-Villanova et al., 2006). The role of E3 ubiquitin ligases in response to plant pathogen has also been widely described (Devoto et al., 2003, Mahmut et al., 2003, Tör et al., 2003, Delaure et al., 2006).

HOW DOES UBIQUITINATION REGULATE CELLULAR PROCESSES?

Accumulating evidence makes regulated protein degradation an excellent means to accommodate the versatile requirements of plant cellular signal transduction. According to this is the recognized importance of negative regulation in controlling plant hormonal and environmental responses. The initiation of opposing stimulatory and repressive actions upon the perception of any signal prevents responses to a single stimulatory event from being perpetuated indefinitely and enables the regulation of response capacity. Rapid reiteration of cycles of pathway activation and deactivation is also essential to the measurement of signal strength over time (Hare et al., 2003).

Like other modifications suited to the repression of signaling (e.g. conjugation to regulatory peptides, phosphorylation, acetylation and prenylation), ubiquitination can occur rapidly, but it may also be transient and rapidly reversed by a set of dedicated de-ubiquitination enzymes (DUBs) (Vierstra, 2003). Nonetheless, whereas repression through covalent modification of a signaling intermediate leaves opens the option of its subsequent re-activation, the irreversibility of protein destruction guarantees signal termination. Receptor de-sensitization, such as the Ub/26S proteasome-dependent degradation of phytochrome A (phyA) when etiolated seedlings are irradiated (Sharrock et al., 2002), is an excellent means of down-regulation in response to a stimulus. Current insights into proteolytic regulation indicate numerous variations on the use of protein degradation in modifying signal transmission in plants but all can be grouped in three categories:

1. **degradation of the repressor of the response:** in certain pathways in which the repression of signaling is the default state, the proteolysis of repressors, triggered by perception of the stimulus as a hormone, activates responses (Hare et al., 2003);

An example of regulation by repressor degradation can be found in the signaling pathway of the important plant hormone, auxin, in which the E3 ligase in the hormone receptor, thus hormone takes direct control of the E3 ligase activity and following signals by promoting the ubiquitination and degradation of a set of transcriptional repressors (Dharmasiri et al., 2005; Kepinski et al., 2005; Figure 6). The natural auxin indol-3-acetic acid (IAA) is supposed to be involved in regulation of every aspect of plant life cycle from embryo patterning and growth responses to fruit development (Woodward et al., 2005). In response to auxin a family of transcription factors called auxin respons factors (ARF's) recognize specific promoter elements, characteristics of auxin responsive genes, activating them. In the absence of auxin, the protein products of a family genes

named AUX/IAA directly interact with ARF and inhibit their trascriptional activity (Reed, 2001; Tan et al., 2007). In the presence of auxin AUX/IAA are removed allowing to ARF's to accomplish their transcriptional activity(Zenser et al., 2001). Moreover the transcription of AUX/IAA genes is also activated by auxin providing an efficient negative feedback mechanism to modulate auxin activity (Liscum et al., 2002). Ubiquitination plays a central role in auxin-induced degradation of AUX/IAA protein. In *Arabidopsis* the gene TIR1, a protein belonging to F-Box family, is a positive regulator of auxin response (Ruegger et al., 1998). When auxin level increases a direct interaction between TIR1 and an auxin molecule occurs, allowing TIR1 to interact with AUX/IAA proteins to direct them through 26S proteasome-mediated degradation. After the removal of AUX/IAA proteins ARF factors can induce the expression of auxin responsive genes (Ramos et al., 2001; Gray et al., 2001).

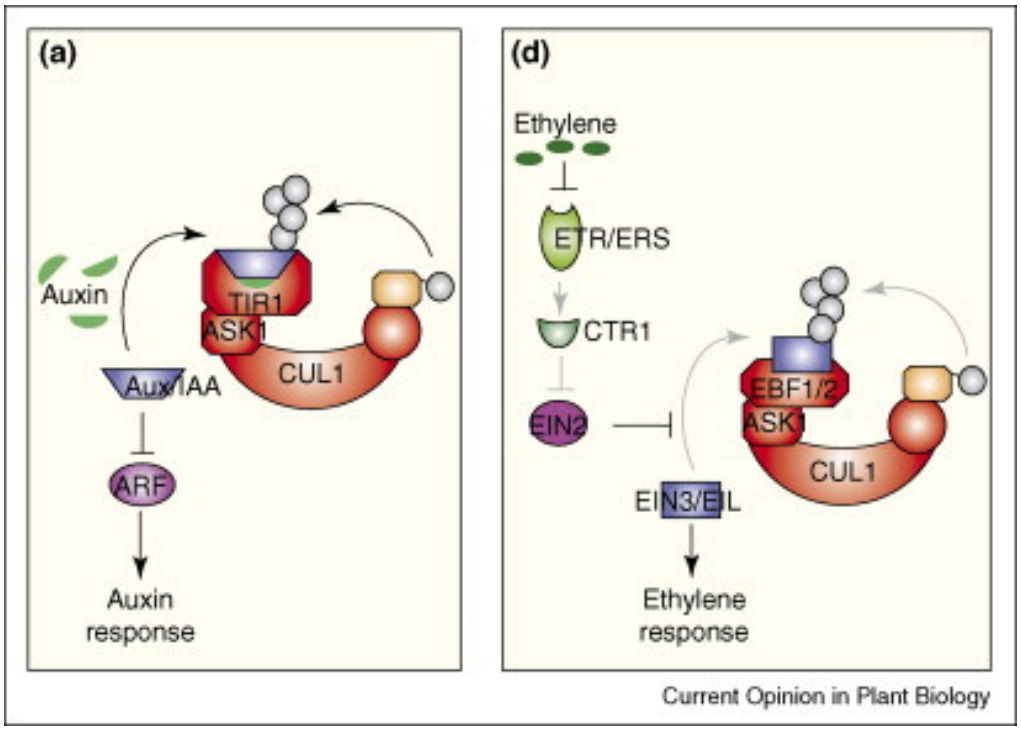


Figure 6: schematic representation of ubiquitin mediated regulation of auxin and ethilene signaling pathway.

2. **degradation of the activator of the response:** in this case the default state is the degradation of the activator; following perception of the stimulus, the degradation is blocked and the signal can be propagated downstream.

The ethylene signaling pathway offers a very peculiar example (Figure 6). Ethylene controls a myriad of growth and developmental processes in plants, including seedling germination and growth, leaf and petal abscission, fruit ripening, organ senescence, and stress and pathogen responses (reviewed in Schaller and Kieber, 2002). Ethylene responses are mediated through a signal transduction cascade that has been partially defined in *Arabidopsis thaliana* using genetic and molecular approaches (Guo and Ecker, 2004; Chen et al., 2005). The hormone perception occurs through ethylene receptors: ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE-INSENSITIVE4 (EIN4), ETHYLENE RESPONSE SENSOR1 (ERS1), and ERS2, which act in a complex involving the Raf-like protein kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1). In the absence of ethylene, CTR1 inhibits downstream events that likely include a mitogen-activated protein kinase (MAPK) cascade. Binding of ethylene to the receptors represses CTR1 action, allowing the MAPK cascade to activates EIN2 (Alonso et al., 1999), which subsequently induces the accumulation of the transcription factor EIN3 (Chao et al., 1997; Ouaked et al., 2003; Yanagisawa et al., 2003). The pathway between CTR1 and EIN3 is also modulated by the EIN5/XRN4 5'-3' exoribonuclease, but its direct RNA target(s) is currently unknown (Olmedo et al., 2006; Potushak et al., 2006).

EIN3 is a key positive switch in ethylene perception, that works by promoting the transcription of a variety of ETHYLENE RESPONSE FACTOR genes (ERF) that ultimately direct the growth and physiological responses under ethylene control (Solano et al., 1998). In addition to EIN3, *Arabidopsis* encodes five EIN3-Like (EIL) transcription factors, EIL1 to EIL5, that may also

contribute to ethylene signaling (Chao et al., 1997; Wang et al., 2002;). EIN3 and EIL1 levels are kept low by selective ubiquitination of the proteins by two F-Box E3 ubiquitin ligases, EBF1 and EBF2, participating respectively in the SCF^{EBF1} and SCF^{EBF2} complexes, which induce their subsequent breakdown by the 26S proteasome (Binderet et al., 2007). In the presence of ethylene, the receptors are inhibited, thus reducing the effect of CTR1 and its subsequent inhibition of EIN2. EIN2 acts in part to directly or indirectly block the interaction of EIN3 and EIL1 with the SCF^{EBF1} and SCF^{EBF2}. The reduction in ubiquitination allows EIN3 and EIL1 levels to rise to mediate ethylene responses. Over a slower time course, EIN2 activation also leads to an increase in EBF1 and EBF2 mRNA and presumably protein levels, which further dampens the accumulation of EIN3 and EIL1. *Via* an unknown mechanism, the exoribonuclease EIN5/XRN4 dampens the accumulation of the EBF1 and EBF2 transcripts. During ethylene signaling, EBF1 plays a special role at no or low hormone levels to maintain low basal levels of EIN3/EIL1. By contrast, EBF2 accumulates during ethylene signaling to prevent excess accumulation of EIN3/EIL1 and to remove EIN3/EIL1 after ethylene levels dissipate (Binder et al., 2007).

3. **attenuation of signal transduction** by targeting different signaling proteins in the same pathway.

It ensures selective changes in the sensitivity of only appropriate subsets of responses, with widespread regulatory consequences. For example, the already mentioned SCF^{TIR1}-mediated degradation of AUX/IAA proteins activates the response but in the same pathway the response can be attenuate downstream by SINAT5 (SEVEN-IN-ABSENTIA OF ARABIDOPSIS THALIANA 5)-mediated degradation of NAC1 (NO APICAL MERISTEM/ATAF-1/CUP-SHAPED COTYLEDO-NLIKE1), a non ARF element involved in lateral root formation.

The importance of combinatorial control (employing a fairly restricted number of factors in different permutations to generate a spectrum of different outputs) has been shown to account for much of the complexity in eukaryotic transcriptional regulation. At the simplest level, this principle is also evident in the vast number of RING and F-box proteins that have an assortment of domains that define their capacity to bind E3 substrates or regulators (Hare et al., 2003). The concept of modularity is further reflected in the design of multi-use core ubiquitination complexes, which comprise both shared elements (e.g. cullins, SKP1-homologs and RBX1 in plant SCF complexes) and proteins that confer specificity. The use of multimeric complexes offers almost infinite possibilities for subtle changes in the sensitivity and specificity of signaling through regulated protein destruction. Regulated participation of substrates in multiprotein complexes not only might bring them into proximity with proteasomes but also may mask or expose domains that are required for ubiquitination. RING proteins of SINAT5's class (Xie et al., 2002) can bind to complexes comprising a SKP1 homolog and a F-box protein through interaction with the adaptor protein SIAH-INTERACTING PROTEIN (SIP) (Matsuzawa et al., 2003), which apparently supplants the scaffolding and regulatory functions of cullin and RBX1. However, the precise roles of E3 components such as RAR1 and plant SIP and SGT1 homologs remain unclear.

The subcellular localization of components of the macromolecular ubiquitination and degradation machines likely plays an important regulatory role. The RING proteins COP1 (Moller et al., 2002) and HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1) (Lee et al., 2001) and the U-box proteins PHOR1 (Amador et al., 2001) and ARC1 (Stone et al., 2003) all accumulate differentially in the nucleus or cytosol in response to a signal that

they transduce. Consistent with convincing evidence of nuclear ubiquitination events, several generic components of the proteolytic apparatus are localized in the nucleus of cells in *Arabidopsis* roots and shoots. SCF and proteasome components appear to co-localize with mitotic spindles and phragmoplasts in actively dividing cells (Farras et al., 2001). Within the nucleus, the existence of aggregates that contain both COP1 and its protein targets suggests that these nuclear bodies may be specialized sites of proteolysis (Holm et al., 2002; Seo et al., 2003). Accordingly, failure of a phyA missense mutant to localize in nuclear bodies was associated with its enhanced stability (Yanovsky et al., 2002). Several generic components of the ubiquitination and proteolytic apparatus appear to accumulate in nuclear bodies (Farras et al., 2001). Convincing evidence of a role for nuclear compartmentation in protein turnover, however, awaits the purification and molecular characterization of these nuclear aggregates.

Developmental, environmental and tissue-specific contexts may significantly influence the relationships between E3 ligases and their substrates. A single substrate might thus be targeted by more than one E3 ligase, although physical or temporal separation might preclude the association of E3s with substrates under conditions in which proteolysis is inappropriate. Conversely, a single E3 might ubiquitinate several substrates *in vivo* through interdependence on regulatory ancillary proteins that influence the E3 catalytic potential. In this regard, current insight into COP1 action provides a useful paradigm: SPA1 apparently stimulates residual nuclear COP1 activity in the light, whereas when the regulation of COP1 abundance alone might impact negatively on other COP1 targets, the direct binding of cryptochromes apparently inactivates COP1 (Yang t al., 2000, Wang et al., 2001).

Finally, the physiological significance of the ability of E3s to self-destruct through auto-ubiquitination, and whether this may be regulated, is not clear. At

least one plant SCF complex is regulated by proteasomal destruction of the F-box protein (Kim et al., 2003). Covalent modification of E3 targets with tags other than Ub likely provides another excellent means of coordinating Ub chain assembly with the needs of cellular physiology. Phosphorylation usually appears to promote substrate degradation (Hare et al., 2003), although it may also stabilize E3 substrates (Hardtke et al., 2000). Links between kinase/phosphatase activities that are associated with specific responses and components of the ubiquitination apparatus may fill in some gaps in transduction chains that are not understood completely.

Perhaps the greatest impediment to the functional dissection of the battery of plant gene products that participate in proteolysis is the likely redundancy between members of large gene families. In this regard, the use of genome expression profiling to define the specificity of E3 expression in different tissue-types and at different developmental stages should narrow down the numbers of potentially redundant candidates in particular responses. Smaller sets of potentially redundant E3s can be studied further using double-mutant analyses. The roles of certain redundant gene products in ubiquitination might also be revealed through the identification of gain of- function mutations that could be identified through classical mutagenesis or activation tagging. Variation in E3 ligase activities can be achieved through changes in the expression of the corresponding mRNAs (Mazzucotelli et al., 2006), induction of multiple splice variants (Mastrangelo et al., 2005, Gingerich et al., 2005), miRNA-mediated gene silencing (Sunkar et al., 2007) and phosphorylation (Pedmale et al., 2007). In addition, given that ubiquitination and sumoylation recognise the same lysine, sumoylation can prevent the protein degradation (Bossis et al., 2006). The E3 ligase activity can also be enhanced by conformational changes due to binding of specific ligands as already mentioned for example for auxin and ethylene.

MOLECULAR MECHANISMS OF PLANT TOLERANCE TO STRESS

From: ***Candidate Genes and QTLs for Barley Adaptation to the Environment,*** *(Tondelli, 2007)*

The research studies at molecular level of the last two decades has led to the identification of many components at the three different steps of the plant response to stress allowing the definition of the various regulatory networks and downstream effectors constituting the tolerance response. Moreover, evidence obtained by these studies indicates that further levels of changes beyond transcriptional ones are activated following exposure of plants to stress and that they are fundamental in the regulation of plant response to stress. Kinases and phosphatases belonging to the signalling cascades activated upon stress have been frequently found upregulated by stress. Analyses of the activation mechanisms of stress related transcription factors have indicated the involvement of post-transcriptional and post translational events. For instance, some stress-related transcription factors have been found activated following a post-translational modification in response to stress signals.

STRESS PERCEPTION

Great interest has been placed in the identification of plant systems for stress perception and in the dissection of the intricate signal transduction pathways responsible for the activation of signalling cascades that confer tolerance to unfavourable conditions (for recent reviews, see Yamaguchi-Shinozaki and Shinozaki, 2006; Guy et al. 2008, Figure 7). However, few pieces of information are today available concerning the existence of a sensing system which can measure the environmental changes, and the list of the identified regulatory components linking receptors to cellular responses is still largely incomplete. In *Arabidopsis* the *ATHK1* gene encoding a transmembrane histidine-kinase is up-

regulated in response to osmotic changes and might transmit the stress signal to a downstream signalling cascade (Urao et al. 1999, Phan Tran et al., 2007, Wohlbach et al., 2008). An early event in the response to many different environmental stresses is an elevation in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) which is used by most cells as a second messenger to convert external signals into cytosolic information (White and Broadley, 2003). To respond appropriately to a specific $[Ca^{2+}]_{cyt}$ perturbation, a cell must activate a unique combination of Ca^{2+}-binding proteins. These $[Ca^{2+}]_{cyt}$ sensors include calmodulins (CaMs), CaM-like proteins, calcineurin B-like (CBL) proteins and Ca^{2+}-dependent protein kinases (CDPKs). For example, a rice gene encoding a calcium-dependent protein kinase (*OsCDPK7*) was induced by cold and salt stresses, and its over-expression enhanced induction of some stress-responsive genes (Saijo et al. 2000). Alternatively, the stress might be sensed through other components either in parallel to or upstream of Ca^{2+} in the pathway; for example, phosphatidic acid (PA) and reactive oxygen species (ROS) seem to play an important role in osmotic transduction pathways (Boundsocq and Laurière, 2005). Moreover, it has been postulated that cold is sensed via changes in membrane fluidity and cytoskeletal reorganization affecting calcium channels (Knight and Knight, 2001). Osmotic stress and the associated oxidative stress appear to be common consequences of exposure to drought, salinity or low-temperature; therefore, it is logical to expect different signaling pathways sharing one or more component. In particular, the above calcium sensors and components of the MAPK cascades might serve as nodes at which cross-talk can occur during the early events of signal transduction. Xiong and Yang (2003) observed that OsMAPK5 expression was induced by ABA, biotic and abiotic stresses and its overexpression in transgenic plants positively regulated drought, salt, and cold tolerance. Also abscisic acid (ABA) plays an important role in the tolerance response of plants to osmotic stress, and some members of the NCED gene family, encoding 9-cis-epoxycarotenoid dioxygenase and involved in ABA

biosynthesis, are strongly induced by drought (Iuchi et al. 2001; Tan et al. 2003). The ABA/receptor interaction produces a rapid increase of secondary messengers such as inositol 1,4,5-trisphosphate (IP3), and cyclic ADP-ribose (cADPR) (Warren et al. 2000). Nevertheless, the role of ABA in cold stress-responsive gene expression is not clear. Several reports have described genes that are induced by dehydration and cold stresses but that do not respond to exogenous ABA treatment. This suggests the existence of ABA-independent, as well as ABA-dependent, signal transduction cascades. The molecular mechanisms regulating gene expression in response to dehydration and cold stresses have been studied by analyzing the cis- and trans-acting elements that function in ABA independent and ABA-responsive pathways.

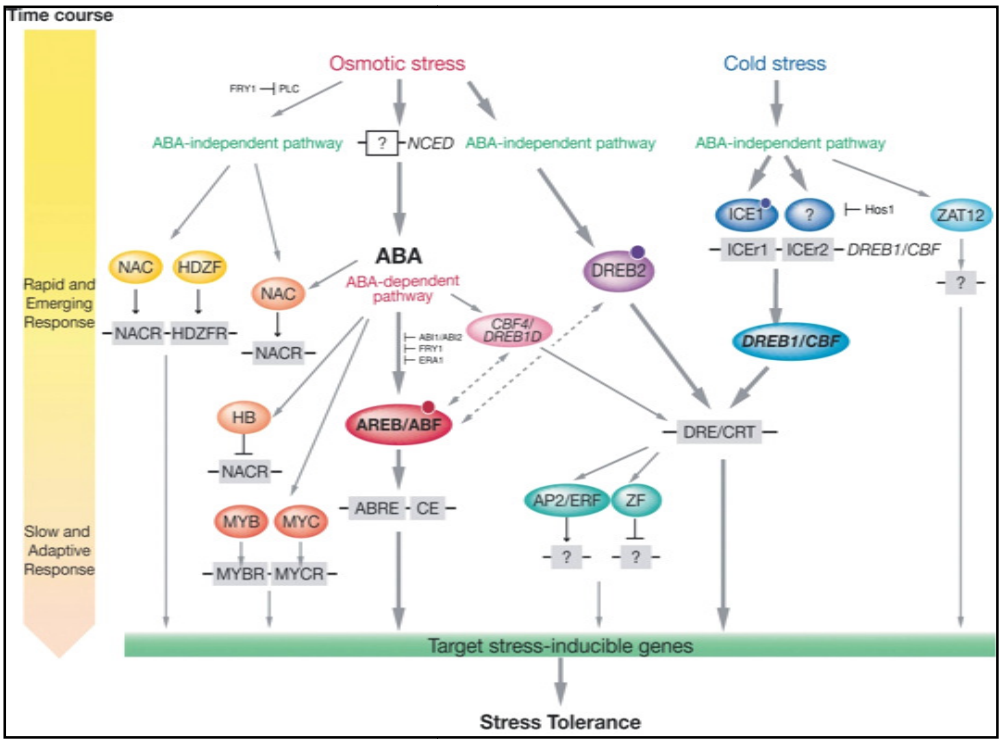


Figure 7: Transcriptional regulatory networks of cis-acting elements and transcription factors involved in osmotic and cold-stress-responsive gene expression in Arabidopsis. (modified from Yamaguchi-Shinozaki and Shinozaki, 2006) Transcription factors controlling stress-inducible gene expression are shown in colored ellipses. cis-acting elements involved in stress-responsive transcription are shown in boxes. Small filled circles reveal modification of transcription factors in response to stress signals for their activation, such as phosphorylation. Regulatory cascade of stress-responsive gene expression is shown from top to bottom. Early and emergency responses of gene expression are shown in the upper part, and late and adaptive responses in the bottom.

TRANSCRIPTIONAL CHANGES UPON STRESS PERCEPTION

In the last decade, the availability of high-throughput system for large transcriptome analysis like microarrays allowed the identification of hundreds of stress inducible genes in different plant species (Rabbani et al. 2003; Maruyama et al. 2004; Buchanan et al. 2005; Svensson et al., 2006; Aprile et al., 2009). These genes are mainly responsible of the last step of the response, coding important metabolic proteins which function in the protection of cells from stress.

They are mainly chaperones, LEA (late embryogenesis abundant) proteins (e.g. barley *af93*; Grossi et al. 1995), osmotin, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis such as proline, water channel proteins, chloroplast-imported proteins (e.g. *cor14b*; Crosatti et al. 1995), sugar and proline transporters, detoxification enzymes, enzymes for fatty acid metabolism, proteinase inhibitors, ferritin, and lipid-transfer proteins. Some of these stress-inducible genes that encode proteins, such as key enzymes for osmolyte biosynthesis, LEA proteins, and detoxification enzymes have been overexpressed in transgenic plants and produce stress-tolerant phenotypes in the transgenic plants (Cushman and Bohnert, 2000). These results indicate that gene products of the stress-inducible genes really function in stress tolerance. These genes products are only the result of the activation of complex signal transduction pathway starting from stimulus perception, through signal transduction and leading to gene products themselves.

Besides stress-inducible genes of the downstream response, many stress-regulated genes encode regulatory proteins of the response, like transcription factors or kinases, often constitutively present and overexpressed upon the stress signal. Deepen analyses of transcription factors and their gene targets allowed the identification of transcriptional regulatory networks of cis and trans-acting

elements involved in gene expression following osmotic and/or cold stress responsive, eventually through ABA signal.

The investigation of promoter regions and of sequence-specific DNA binding proteins of ABA regulated genes in *Arabidopsis*, highlighted several DNA *cis*-acting elements. One of these is known as ABRE (ABA-responsive element) (Guiltinan et al. 1990), with its 'G-box' ACGT core element. Promoters containing 'G-boxes' represent the target of the ABRE-binding factors (AREB/ABF) that generally are dimeric bZIP-proteins (with the typical basic leucine-zipper structural motif at the C-terminus). Nevertheless, in many genes, ABRE alone is not sufficient for gene activation, and an additional DNA *cis*-element named coupling element (CE) is required. CEs are binding sites for *trans*-activator proteins that interact with the AREB/ABFs (Shen and Ho, 1995; Shen et al. 1996); moreover AREB1 is shown to be activated by phosphorylation *in vitro* (Fujita et al., 2009). In barley, a basic domain/Leucine zipper (bZIP) transcription factor, HvABI5, is able to recognize the ABA response promoter complexes of the barley *HVA1* and *HVA22* genes and is necessary for the ABA up-regulation of the expression of the two genes (Casaretto and Ho, 2003). Also in this case posttranslational modification play a key role in regulating Transcription factor activities; the *Arabidopsis* ABI5 was shown to undergoes SIZ1 mediated sumoylation (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001; Lopez-Molina et al., 2002) Besides AREB/ABFs, other transcription factors are known to control the ABA-dependent expression of stress-related genes. In *Arabidopsis* both a MYB- and a MYC-transcription factors are synthesized following accumulation of endogenous ABA and have been shown to bind respective *cis*-elements in the promoter region of the drought-inducible gene *rd22* (Abe et al. 2003). In rice, the OsMYB4 transcription factor is strongly induced by different abiotic stresses, and the expression of Cold-Regulated (COR) genes is affected in *OsMYB4* transgenic plants. As these MYB- and MYC-transcription factors act cooperatively in the

late stage of stress response, they could be involved in the slow and adaptive stress response process to dehydration (Yamaguki-Shinozaki and Shinozaki, 2006).

A variety of stress-related genes are induced by both dehydration and low-temperature, but are not subjected to ABA control. The analysis of the promoter regions of these genes led to the identification of a DNA cis-acting element named DRE (dehydration-responsive element) characterised by the consensus sequence TACCGACAT (Yamaguchi-Shinozaki and Shinozaki, 1994). The cis-acting core sequence of the DREs (CCGAC) is also known as CRT (C-repeat) (Baker et al. 1994). Transcription factors belonging to the ERF/APETALA2 (AP2) family that bind to DRE/CRT have been firstly isolated in *Arabidopsis* and termed DREB1/CBF (C-REPEAT-BINDING FACTOR) and DREB2, according to their aminoacid sequence (Stockinger et al. 1997; Gilmour et al. 1998). DREB1/CBF proteins are induced by cold stress, while DREB2 factors are regulated by dehydration (Liu et al. 1998), indicating the existence of two parallel signal transduction pathways in drought and cold stress, converging on the same DRE/CRT cis-acting element.

The evidence that some molecular mechanisms involved in stress tolerance are conserved between species also phylogenetically distant, and the availability of a vast amount of genetic information in public database supply the scientific community with powerful tools for comparative genomics and for transfer of knowledge from model species to crop (Cattivelli et al., 2008). After the identification of DRE/CRT elements in *Arabidopsis*, similar responsive elements have been found to control the stress-related expression in many different plant species. DRE-like elements have been found in the promoter regions of drought-inducible genes such as *rab17* in maize (Busk et al. 1997), *wsi18* in rice (Joshee et al. 1998) or *HVA1* in barley (Straub et al. 1994). As a consequence, *CBF*-like sequences have been isolated on the basis of homology

with *Arabidopsis* counterparts in different species including cereals such as barley and wheat (Gao et al. 2002, Choi et al. 2002; Xue 2002). Four *OsDREB1s* and one *OsDREB2* have been identified in the rice genome sequence and have been found to function in stress-inducible gene expression (Doubuzet et al. 2003). Overexpression of *OsDREB1A* in *Arabidopsis* revealed that this gene has a similar function to that of its *Arabidopsis* homolog in stress-responsive gene expression and stress tolerance. On the other hand, stress-induced expression in wheat of the *Arabidopsis DREB1A/CBF3* gene has been shown to delay water stress symptoms (Pellegrineschi et al. 2004), and a set of *COR* effector genes is trans-activated by barley *HvCBFs* (Xue 2002, 2003; Skinner et al. 2005). This suggest that the cold responsive pathway triggered by these transcription factors is conserved between dicotyledons and monocotyledons.

Cold-responsive *DREB1/CBFs* expression studies revealed interesting features about their regulation, in particular it highlighting the critical role of negative regulation. It could represent a general paradigm used by plants to control other cellular processes. This regulatory mechanisms guarantee both the proper induction of downstream genes and the accurate development of freezing tolerance, ensuring that *DREB1/CBFs* expression is transient and tightly controlled:

- the steady-state levels of CBF transcripts increase in response to cold shock and gradual temperature downshifts,
- the cold-sensing mechanism becomes desensitized with time at low temperature,
- the CBF transcripts have a very short half-life at warm temperature (Zarka et al. 2003). Moreover, it was recently demonstrated that the expression of *DREB1B/CBF1* and *DREB1A/CBF3* in response to cold preceded that of *DREB1C/CBF2*, which, in turn, negatively regulates CBF1 and CBF3 (Novillo et al. 2004).

Upstream of DREB1/CBFs, the *INDUCER OF CBF EXPRESSION1* (*ICE1*) gene was identified through the map-based cloning of the *Arabidopsis ice1* mutation (Chinnusamy et al. 2003). *ICE1* encodes a MYC-type bHLH transcription factor that regulates the expression of *DREB1A/CBF3* but not of other *DREB1/CBF* genes and its overexpression resulted in improved freezing tolerance. Moreover, molecular analysis of the *DREB1C/CBF2* promoter has identified two cis-acting elements designed *ICEr1* and *ICEr2* (*Induction of CBF Expression region 1* and *2*) that are involved in their cold-inducible gene expression (Zarka et al. 2003). The cloning of the corresponding DNA-binding protein has identified a MYC-type bHLH transcription factor different from ICE1, suggesting again a redundant involvement of these transcription factors in the upregulation of *DREB1/CBF* genes (Shinozaki et al. 2003).

A unique mutant screening system using transgenic *Arabidopsis* plants with a firefly LUC reporter gene under the control of the *rd29a* promoter has been developed to screen mutants that have defects in their abiotic-stress signal transduction pathways (Ishitani et al. 1997). Using this system, many *Arabidopsis* mutants have been isolated that have altered induction of stress-responsive genes by drought, high-salinity, cold and ABA. These mutants exhibited altered expression of the *rd29a::LUC* gene at a constitutive (*cos*), high (*hos*), or low (*los*) level in response to various abiotic-stress or ABA treatments (Ishitani et al. 1997). The mutated genes are thought to function directly in the induction of *rd29a* as well as upstream the *rd29a* induction in signal transduction pathways and also in posttranscriptional regulation of the activation of the DREB1/CBFs, DREB2s and/or AREB/ABFs transcription factors (see Zhu, 2002 and Xiong and Schumaker, 2002 for recent reviews). For instance, as the *hos1* mutation enhances the stress dependent induction of DREB1/CBF factors HOS1 was thought a negative regulators of the cold signal transduction functioning in the degradation of CBF/DREB1 and ICE proteins (Lee et al, 2001). As it is located in the cytoplasm but moves to the nucleus upon cold

treatment to control the expression of *CBF/DREB1,* a post-translational modification could mediated its change of subcellular localization.

THE ROLE OF ALTERNATIVE SPLICING AND UBIQUITINATION IN THE MOLECULAR RESPONSE TO STRESS

From ***: Abiotic stress response in plants: When post-transcriptional and post-translational regulations control transcription*** *(Mazzucotelli, Mastrangelo, Crosatti, Guerra, Stanca, Cattivelli, 2008)*

STRESS-RELATED TRANSCRIPTS FROM ALTERNATIVE SPLICING EVENTS

Alternative splicing is a mechanism by which multiple forms of mature mRNAs are produced from a single transcript, just after the transcript synthesis. The mRNAs of about 35–60% of human genes are considered to be alternatively spliced (Nagasaki, et al., 2005), while between 20 and 60% of plant genes, depending on the species considered, give rise to mRNA that are alternatively spliced (Ner-Gaon et al., 2007). Four main types of alternative splicing are known: exon skipping, alternative 5' and 3' splice sites and intron retention. Often, events of the first three alternative splicing types lead to functionally relevant changes in the protein products, such as replacement of the amino or carboxy terminus, or in-frame addition/removal of a functional unit. In this way, different polypeptides, with different functions or subcellular locations, are produced by a single gene. Exon skipping is the most frequent and intron retention the rarest alternative splicing form in animals (Kim, et al., 2007), while intron retention is the most common alternative splicing in *Arabidopsis* and rice (>50%; Kim, et al 2007, Ner-Gaon et al., 2004). mRNAs with introns lead to truncated polypetides, or are subjected to nonsense-mediated mRNA decay, as

retained introns often introduce in frame stop codons (Maquat, et al., 2004). Nevertheless, the high rate of intron retention in plants and numerous studies available in literature suggest that it may represent the result of an active process inhibiting the splicing reaction rather than inaccurate intron recognition. The production of truncated, inactive transcripts at the expense of the corresponding full length mRNA, can work as a system regulating the amount of the active protein form finally produced, probably due to the lower level of correctly spliced transcript, or competition of different forms for association with ribosomes (Maquat, et al., 2004, Quesada, 2003). In addition, the shortened polypeptides following alternative splicing are not necessarily functionless forms of the full length protein, as shown for the N gene for resistance to tobacco mosaic virus where both full length and shortened alternative transcripts are needed for the complete resistance phenotype (Dinesh-Kumar et al., 2000).

Alternative splicing events do not randomly affect mRNA of all genes, rather they seem to occur preferentially to mRNAs of certain classes of genes commonly involved in signal transduction, or encoding enzymes, receptors and transcription factors (Ner-Gaon et al., 2006, Lareau, et al., 2004). In plants some transcription factors undergo splicing in response to environmental constraints. The wheat transcription factor gene Wdreb2 generates three stress-regulated transcripts through exon skipping mechanism, in response to cold, drought, salt and exogenous ABA treatments. The three forms have different accumulation profiles and their expression is regulated through an ABA-dependent pathway during drought and salt stresses and an ABA-independent one under low temperature. Notably, the absence of second and third exons in one of the three transcripts do not impair its activity as transcriptional activator of downstream stress-related genes, like cor genes and LEA protein genes, in a yeast assay (Egawa, et al., 2006). A subgroup of MYB transcription factor genes produces alternative transcripts whose accumulation is dependent on phytohormones and stress conditions in *Arabidopsis* and rice. Through alternative splicing,

AtMYB59 and AtMYB48 genes are able to encode putative proteins differing for their MYB repeats and probably for their binding affinities to gene promoters (Li, et al., 2006). Genes encoding proteins involved in posttranslational modifications and signal transduction cascades may also be regulated by alternative splicing. The mRNA of a durum wheat gene encoding an ubiquitin ligase retains the 3'UTR-located intron in response to cold and dehydration stress (Mastrangelo, et al., 2005) The rice gene OsBWMK1, encoding a MAP kinase, has three transcript variants: OsBWMK1L, OsBWMK1M and OsBWMK1S. The second and third ones are induced by various stresses, while OsBWMK1L is constitutively expressed. Proteins deriving from the three transcripts are characterized by differential subcellular localizations: OsBWMK1S is primarily in the nucleus, while OsBWMK1L and OsBWMK1M are predominantly in the cytoplasm. Nevertheless, a treatment with defense signaling related molecules determines the translocation of OsBWMK1L from cytoplasm to nucleus (Koo, et al., 2007).

The serine/arginine proteins are a class of RNA binding proteins with a role in splicing control. They are known to promote alternative splicing of their own transcripts as well as of other gene products (Wollerton, et al., 2004, Wang, et al., 2006). Nineteen genes encoding serine/arginine proteins have been identified in *Arabidopsis*, and most of their mRNAs undergo alternative splicing following developmental and environmental stimuli producing 95 different transcripts (Isshiki, et al., 2006, Palusa, et al., 2007). The regulation by alternative splicing of genes whose products in turn alter the splicing of other genes may considerably enhance and amplify the signal transduction cascade in response to stress stimuli. The *Arabidopsis* STA1 gene encodes a pre-mRNA splicing factor up-regulated by cold. The analysis of sta1-1 mutant shows that STA1 protein can regulate the stability and splicing pattern of a number of endogenous gene transcripts related to abiotic stress response (Lee, et al., 2006). In particular, the sta1-1 mutant is characterized by the non-correct splicing of

cor15a mRNA, a messenger encoding a cold-induced chloroplast-targeted polypeptide. The finding that over-expression of cor15a enhances the in vivo freezing tolerance of chloroplasts in nonacclimated plants can therefore explain the chilling sensitivity of the sta1-1 mutant (Artus, et al., 1996).

Although only few alternative splicing events have been described so far in response to abiotic stresses, the recent findings indicate that a large proportion of the genes undergoes alternative splicing (Ner-Gaon et al., 2007). Therefore the effect of alternative splicing on the stress related transcriptome is probably still underestimated and a number of not yet known alternative splicing-based mechanisms are likely to play a role in the tolerance to abiotic stresses.

PROTEIN DEGRADATION IN RESPONSE TO STRESS: THE ROLE OF UBIQUITINATION

A number of studies have pointed out the relevance of the ubiquitin-dependent protein degradation in stress-related signalling and response mechanisms (Ellis et al., 2002, Zhang et al., 2007, Zhu et al., 2007). Transcriptome and proteome analyses carried out in different plant species following exposure to abiotic stresses indicated that hundreds of ubiquitination-related transcripts/proteins are modified during stress responses suggesting a role for ubiquitination in determining the stress tolerance (Mazzucotelli et al., 2006, Zang et al., 2007, Dooki et al., 2006, Pandey et al., 2008). This is supported by the recent proteomic analysis of the flower buds of *Arabidopsis* plants carrying a mutation in the ASK1 gene, a critical component of the SCF ubiquitin ligase complexes. In ask1, the impairment of the SCF ligase-mediated ubiquitination and the resulting accumulation of SCF targets allowed the identification of ubiquitination targets, among them a number of stress-related

proteins (Wang et al., 2006). Some E3 ubiquitin ligases regulate stress response acting both in hormone dependent and in hormone independent pathways.

Recent findings indicate that the protein degradation mediated by the ubiquitin/proteasome pathway plays a fundamental role in ABA homeostasis and response. The H2-type zinc-finger protein SDIR1 is a positive regulator of ABA signalling, acting upstream of the main transcriptional regulators of the ABA molecular response (Zhang, et al., 2007). Indeed, the up-regulation of SDIR1 gene expression enhances ABA-induced stomatal closure resulting in increased drought tolerance. In crosscomplementation experiments, the ABA-insensitive phenotype of the sdir1-1 mutant can be rescued by several transcription factor genes acting in the ABA pathway (ABI5, ABF3 and ABF4). Several RING-type E3 ligases are involved in the ABA-dependent molecular responses. Notwithstanding, the up-regulation of the XERICO gene, encoding a H2-type zinc-finger E3 ubiquitin ligase, results in increased drought tolerance due to an enhanced ABAinduced stomatal closure (Ko, et al., 2006). XERICO controls the level of ABA by enhancing the transcription of the key ABAbiosynthetic gene AtNCED3. XERICO also interacts with AtTLP9, an E3 TUBBY ligase acting as positive regulator of ABA signalling (Lai, et al., 2004). The U-Box-containing E3 ubiquitin ligase AtCHIP was showed to be involved in ABA mediated stress response (Luo et al., 2006). AtCHIP interacts and ubiquitylate the A subunit of protein phosphatase 2 (PP2A) that regulates activity of several important enzymes involved in carbon metabolism during stress response. Surprisingly PP2A protein were not degraded by AtCHIP, but the stability of the protein increase follows the ubiquitination. Moreover *Arabidopsis* plant overexpressing AtCHIP are more sensitive to ABA treatment (Luo et al., 2006). The *Arabidopsis* RING finger E3 ligase RHA2a is also a positive regulator of abscisic acid signaling during seed germination and seedling development. Moreover, consistently with these data RHA2a negatively regulates seed germination on salt medium (Bu et al., 2009).

Some E3 ubiquitin ligases have been shown to regulate stress response acting in hormone independent pathways. The already mentioned HOS1 is responsible of the ubiquitination of the key regulator of cold stress response ICE1, the transcription factor Inducer of CBF Expression 1 (Dong et al., 2006). HOS1 encodes a RING-finger protein E3 ubiquitin ligase which exerts a negative control on cold response (Ishitani, et al., 1998). Indeed HOS1 mediates the ubiquitination of ICE1, leading to its proteasome-mediated degradation during exposure to cold. According to this function, hos1 mutation enhances the induction of CBFs and of the downstream cold-regulated genes by low temperatures (Dong, et al., 2006). A RING ubiquitin ligase is also responsible of the control of the master regulator DREB2 of drought response. By a yeast two-hybrid screen a protein interacting with the drought-induced transcription factor DREB2A were identified and named DRIP1 (DREB2A-interacting protein1; Qin et al., 2008). *DRIP1* codes for a C3HC4 RING domain-containing protein. DRIP1 is an active E3 ubiquitin ligase non-stress induced able to mediate the ubiquitination of DREB2A. Overexpressed of *DRIP1* in *Arabidopsis* plants delays the expression of drought responsive genes, on the other side *Arabidopsis drip1* mutants show an enhanced expression of stress responsive genes and an enhanced tolerance to dehydration stress. This results strongly suggest the role of *DRIP1* as a negative regulator of drought stress response (Qin et al., 2006).

In recent work (Lee et al., 2009), another drought stress-induced RING finger protein, Rma1H1 for RING membrane-anchor 1 homolog 1, was isolated in *Capsicum annuum* and characterized through cross transformation in *A. thaliana*. Expression analysis on Rma1H1 showed a clear induction during drought stress with a major amount of transcript in leaf tissue. Rma1H1 is also induced by cold stress, mechanical wounding, high salinity and ethylene but not by ABA. Moreover Rma1H1 confer drought tolerance when overexpressed in *Arabidopsis* plants (Lee et al., 2009).

Evidence is accumulating about reciprocal actions among different kinds of transcriptional, post-transcriptional and post- translational regulations. The emerging picture is an increasing variety of interacting mechanisms shaping the transcriptome and proteome and contributing to the fine tuning of cell metabolism (Figure 8).

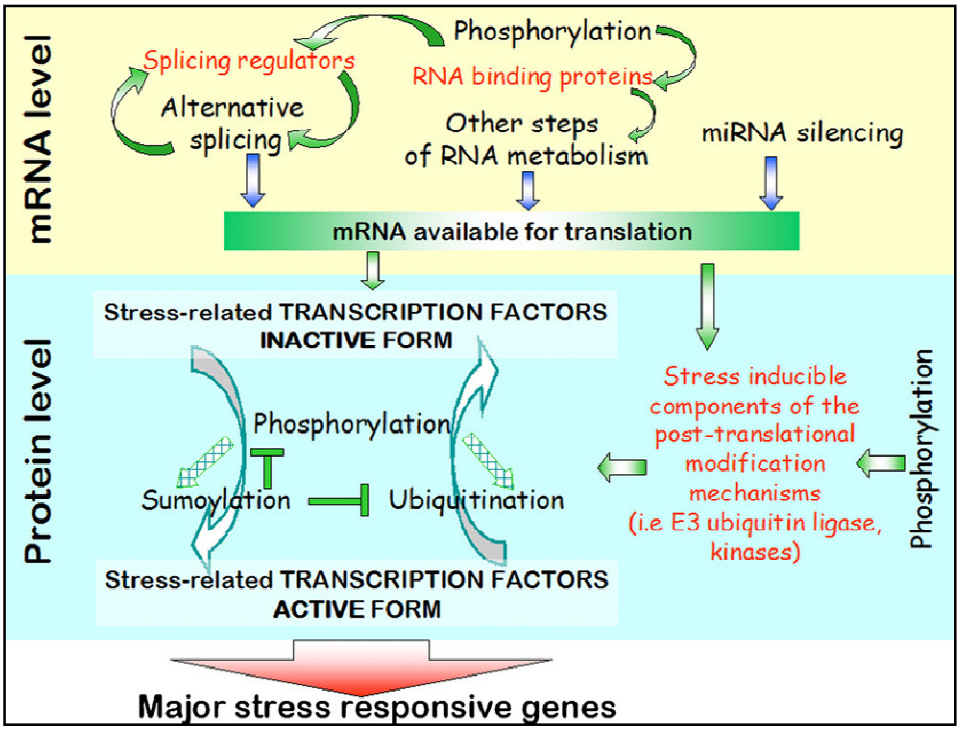


Figure 8: Model describing the cross-talking among post-transcriptional (mRNA level) and post-translational (protein level) regulations involved in the control of the plant response to abiotic stress. See the text for details.

The expression of genes encoding components of the posttranslational control is often controlled at transcriptional level (i.e. many E3 ubiquitin ligases are stress induced), subjected to gene silencing by action of miRNA (Sunkar et al., 2004) or to alternative splicing events (Mastrangelo et al., 2005).

Furthermore the corresponding proteins might be phosphorylated (Lee et al., 2003). Perusing lists of potential kinase substrates reveal intriguing connections between posttranscriptional mechanisms and phosphorylation. Splicing factors, RNA helicases as well as transcription factors were also among the targets of the stress-related MAP kinase3 and MAP kinase6 (Feilner et al., 2005). In *Arabidopsis* 79 unique phosphorylation sites were identified in 22 phosphoproteins having a role in RNA metabolism and mRNA splicing, including RNA helicases. As among them were some spliceosome SR proteins involved in hormone and abiotic stress response, the activation of specific splicing factors by phosphorylation during the exposure to abiotic stresses can be hypothesized (Lee et al., 2006, van Bentem et al., 2006).

Conversely, alternative splicing can also control protein phosphorylation. The rice gene OsBWMK1, encoding a MAP kinase, produces three protein variants based on alternative splicing events, two of them in response to various abiotic stresses (Wollerton et al., 2004). A link between RNA processing and SUMO modification has been also recognized, in which SUMO pathway can be a possible mechanism to control nucleocytoplasmic transport of proteins (Pichler et al., 2002). Besides many hnRNPs, RNA helicases, and other proteins of RNA metabolism identified as substrates for SUMO modification in mammals (Vassileva et al., 2004), in plants a mutant in a nuclear pore protein, NUA, is affected both in SUMO homeostasis and nuclear RNA accumulation (Xu et al., 2007).

Multiple signaling pathways may converge on the same target protein by multisite modifications, resulting in complex combinatorial regulatory patterns that dynamically and reversibly affect the activity of a target protein. Different post-translational mechanisms may act together or have antagonistic effects. In animals, phosphorylation of a protein target is often essential to its ubiquitination (Pedmale et al., 2007). For example, a whole class of F-box

subunits of SCF ubiquitin–protein ligases binds to and thus recognizes phosphorylated epitopes on their substrates (Karin et al., 2000). Sumoylation and phosphorylation reciprocally interact on the target proteins, with sumoylation only targeting phosphorylated proteins, or preventing phosphorylation (Bossis et al., 2006). In addition, ubiquitination and sumoylation often have antagonistic effects by acting on the same amino acid residues (Johnson, 2004).

Understanding how different modifications act on the same target as well as the in vivo modalities and timings of these interactions, is a future challenge for the understanding of plant responses to abiotic stresses. Evidence about these networks in plants is still limited. However some recent insights on regulation of the activity of the transcription factor ICE1 offer a well characterized example of the complexity of these regulatory systems. ICE1 is constitutively expressed, nevertheless it activates the expression of CBF genes only upon cold treatment (Chinnusamy et al., 2003). Three different modifications are known, so far, to control the activity of ICE1 protein. At low temperature ICE1 can undergo sumoylation through the action of AtSIZ1 (Miura et al., 2007), resulting in a fully active transcription factor. Alternatively HOS1 can cause ubiquitination of ICE1 and consequently its proteosomal degradation (Dong et al., 2006). ICE1 may be more or less available for ubiquitination and sumoylation depending on the protein phosphorylation status, which is most likely temperature dependent (Zhu et al., 2007). Similarly we can hypothesize a nuclear cold-induced localization of HOS1 by phosphorylation. The balance between activation and degradation allows a perfect tuning of ICE1 activity which in turn leads to the activation of the cold-induced molecular response. The signalling pathway controlling the phosphate homeostasis represents an example of how a cascade of different regulatory mechanisms can regulate the final expression of stress-related genes. The MYB transcription factor PHR1 is post-translationally regulated by the SUMO E3 ligase AtSIZ1 (Miura et al., 2005). PHR1 is

involved in the induction of miR399 in response to phosphate deprivation. The accumulation of miR399, in turn, represses the PH2 gene encoding the ubiquitin conjugating enzyme UBC24 (Bari et al., 2006). The final effect is, presumably, the attenuation of an ubiquitin pathway that negatively regulates the expression of phosphate transporters and root growth in normal conditions, maximising phosphate uptake during starvation.

Even if still speculative, interactions among post-transcriptional and post-translational regulations can be expected in the epigenetic component of the stress tolerance. Molecular mechanisms underpinning epigenetics include modification of histones and chromatin remodeling, besides DNA modification (Loidl, 2004). Many post-transcriptional and post-translational regulations are involved in epigenetic changes. The final effect of RNA-mediated gene silencing is often the methylation of the genomic region producing the target RNA. Phosphorylation, ubiquitination and sumoylation beyond acetylation and methylation, act on nucleosome core histones and sumoylation regulates the activity of the chromatin remodeling complexes (Fuchs et al., 2006). All together these modifications constitute a histon code which activates or silences gene expression by modifying chromatin structure. Epigenetic changes have been implicated in the acclimation process, a phenomenon that allows a plant to become more resistant to future stress exposure after a previous stress sensing (Bruce et al., 2007). We believe that further progress on the understanding of the epigenetic contribute to stress tolerance will reveal new insights on the role of non-transcriptional regulations.

POST-TRANSCRIPTIONAL AND POST-TRANSLATIONAL REGULATIONS: FUTURE CHALLENGES FOR THE UNDERSTANDING OF THE PLANT RESPONSE TO ABIOTIC STRESSES

The recent progress of knowledge on plant abiotic stress response is depicting a frame where mechanisms controlling mRNA availability and protein activity act together to finely and timely adjust transcriptome and proteome to the continuous variations of environmental conditions. Future successful strategies to advance knowledge on plant responses to abiotic stresses will concern the functional characterization of key cellular regulators by genetic analyses of the corresponding mutants as well as by transcriptome and proteome surveys on transcriptome complexity, protein–protein interactions and post-translational modifications of proteins. These outcomes will lead to the identification of new environmental related pathways as well as of their target molecules. Though not yet documented in the context of the plant response to abiotic stress, knowledge from other organisms and experimental systems suggests that post-transcriptional and post-translational regulations are able to integrate external signals. For example, the activity of ubiquitination in the regulation of development processes is triggered by developmental hormones. Sensing of auxin is accounted directly by the F-box protein of an E3 ligase, TYR1 (Tan et al., 2007), while gibberellins bind to a protein that, in turn, associates with an E3 ligase (Ueguchi-Tanaka et al., 2007). Intriguing indications come also from plant defence responses to biotic stresses. The RNA mediated silencing is directly activated by virus nucleic acids (Lindbo et a., 2005) and alternative transcripts of some resistance genes are required for rapid and complete R gene-mediated resistance (Dinesh-Kumar et al., 2000, Zhang et al., 2007). Moreover, ubiquitination in the defence response is directly triggered by jasmonic acid, the crucial plant hormone of host immunity (Thines et al., 2007). These specific events may underline a more general situation where

developmental- and environmental-related signals are integrated in the regulatory pathways controlling plant responses through post-transcriptional and post-translational regulation.

AIM OF THE WORK

The screening of a suppression subtractive library developed from leaves of durum wheat seedlings exposed to low temperature for 6h led to the isolation of some early cold-regulated (*e-cor*) genes including a sequence encoding for a putative E3 ligase, *TdRiSR* (RING-finger protein Stress Related, described as *6g2* in Mastrangelo et al., 2005). Beside cold stress, the e-cor gene *TdRiSR* is up-regulated both by fast dehydration and drought and undergoes cold-induced intron retention of the fourth intron (De Leonardis et al., 2007, Mastrangelo et al., 2005).

After cloning a specific work was undertaken to study the cellular function of *TdRiSR* gene product with specific attention to its role in the plant response to abiotic stress and with the final aim to:

- verify if TdRiSR protein possesses E3 ubiquitin ligase activity by *in vitro* ubiquitination assay;
- determine subcellular localization of TdRiSR by transient transformation of onion epidermal cells;
- identify protein interactors by means of two-hybrid system after developing an expression library;
- investigate the involvement of TdRiSR in protecting cells against fast dehydration;
- develop a new system for identifying E3 ligases target on whole proteome in plants.

MATERIALS AND METHODS

BIOLOGICAL MATERIAL

Plant material

Arabidopsis thaliana ecotype Columbia (Col-0) were used to perform PF2D liquid chromatography system experiments.

7 days-old durum wheat plants Cv. Ofanto were used to prepare mRNA for expression library.

E. coli and yeast strains

- Cloning reaction were performed in *E. coli* strain DH5α.
- Gateway vectors were propagated in *E. coli* strain DB3.1.
- For protein induction *E. coli* strain BL21 pLys Rosetta was used.
- Two-hybrid analyses were performed in yeast strain AH109 (MATa trp1, leu2 which contains ADE2, HIS3, lacZ and MEL1 reporter genes, each using a distinct GAL4-responsive promoter) and yeast strain Y187 (MATα trp1, leu2, which contains the lacZ and Mel1 reporters under the control of two distinct GAL4- responsive promoters)

CULTURE MEDIA

1X LB medium (Luria-Bertani medium)

10 g tryptone
5 g yeast extract
10 g NaCl
SDW to 1L

Adjust the pH to 7,0 with 5 N NaOH. Add 15gl-1 of Difco Bacto Agar if required and Sterilize by autoclaving

1X Murashige and Skoog (MS) medium

4,3g l^{-1} MS salts.
3% (w/v) sucrose.

Adjust the pH to 5,7 with KOH. Add 20gl^{-1} of Difco Bacto Agar for solid medium and Sterilize by autoclaving

Synthetic Complet Drop Out (SC drop-out) medium

4,0 gm Difco Yeast Nitrogen Base (w/o amino acids)
12,0 gm D-glucose
0,50 gm Synthetic Complete Drop Out Mix
SDW to 600 ml

Adjust the pH to 5,6 with 10 N NaOH. Add 12,0 gm Difco Bacto Agar for solid medium and autoclave at 121°C for 15 min.

Depending of the auxotrophy to be tested omit Synthetic Complete Drop Out Mix and add single, double, triple o quadruple drop-out medium the solution. For colorimetric assay spreed X-α-GAL on the surface of SC agar plates. In this work multiple drop out medium and X-α-GAL from Clontech and Sigma were used.

YPAD medium

6,0 gm yeast extract (Difco)
12,0 gm Peptone (Difco)
12,0 gm Glucose
60 mg adenine hemisulphate
600 ml distilled water

Adjust the pH to 6 with 10 N NaOH. Add 12,0 gm Difco Bacto Agar for solid medium and autoclave at 121°C for 15 min.

SOLUTIONS

1X PBS (Phosphate Buffer Saline)

140 mM NaCl,
2,7 mM KCl
10 mM Na_2HPO_4,
1,8 mM KH_2PO_4
SDW (Sterile Distilled Water) as needed
pH 7,3

For experiments in which Tween20 is required, add 1ml of Tween20 to 1l of 1X PBS. For protein extraction add the serine protease inhibitor PMSF (PhenylMethylSulphonyl Fluoride). PMSF is rapidly degraded in water and stock solutions are usually made up in anhydrous ethanol, isopropanol, corn oil, or DMSO. Proteolytic inhibition occurs when a concentration between 0.1 - 1 mM PMSF is used.

2X Laemmli Buffer

4% SDS (Sodium Dodecyl Sulphate)
20% glycerol
10% β-mercaptoethanol
0,004% bromophenol Blue
0,125M Tris-HCl
SDW as required

Ponceau Red Stain

0,5% Ponceau Red
1% Acetic Acid
SDW as required

Blue Coomassie Stain

0,2% Coomassie brilliant Blue R-250
7,5% Acetic Acid
50% Ethanol
SDW as requiered

Lower Buffer

187 g Tris Base, pH 8.8 with HCl
4,0 g SDS
SDW to 1liter

Upper Buffer

60,5 g Tris Base, pH 6.8 with HCl
4,0 g SDS
SDW to 1liter

Blocking solution

1X PBST (PBS-Tween20 0,1%)
5% Non fat Dry Milk or BSA (Bovine Serum Albumin)

10X Running buffer

30,3 g Tris base
144,1 g Glycine
10 g SDS
SDW to 1L

General lysis buffer

6M urea
2M thiourea
10% glycerol
50mM Tris-HCl (pH 7.8-8.2)
2% n-octylglucoside
5mM TCEP (Tris (2-carboxyethyl) phosphine hydrochloride)
1mM protease inhibitor

Vectors

pDNR221	INVITROGEN
pDEST17	J. Cullis
pDEST32	INVITROGEN
pEXP-AD502	INVITROGEN
pMAL	J.C.del Pozo
pGEX-2KT	J.C.del Pozo
pMDC83	Mark D. Curtis

PROCEDURES

Yeast growth conditions

The *S. cerevisiae* cells were grown on standard YPAD medium or SC medium supplemented with the appropriate amino acids (Sigma/Clontech) at 30°C with shaking at 200rpm. The yeast strains were transformed using the modified lithium acetate method (Gietz and Woods 2001).

Wheat cDNA library

For the cold treated cDNA library, seeds of *T. durum L.* cv Ofanto were germinated in pots containing soil 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. Cold treatment was performed by subjecting germinated seedlings to a temperature of

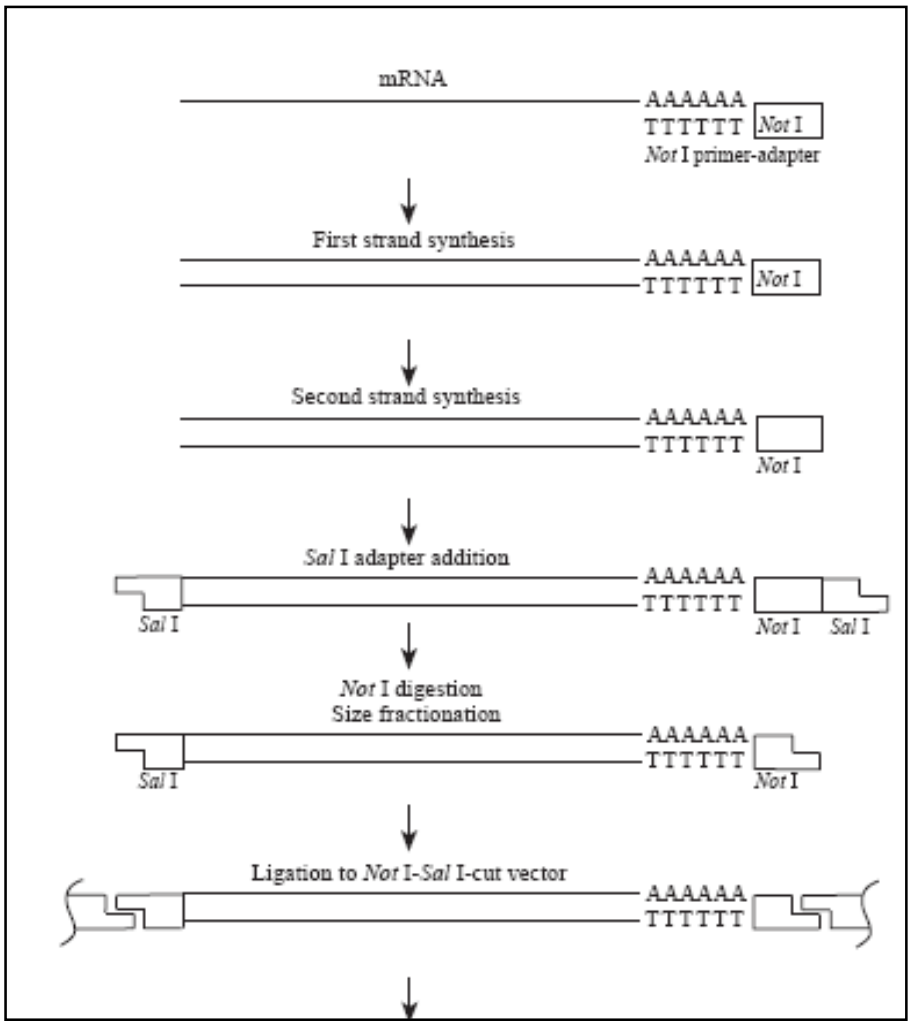
3°C for 6h under an irradiance of 300 µmol m^{-2} sec^{-1}. Leaf were collected and frozen at -80°C. Total RNA from leaf were isolated using the TRIzol® reagent (GibcoBRL).

50-100 mg of Leaf tissue from durum wheat were homogenized in liquid nitrogen and then added with 1 mL of TRIZOL Reagent; 0.2 mL of chloroform per 1 mL of TRIZOL Reagent were added and mixed. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol, washed in 75% ethanol and then resuspended in H_2O-DEPC.

mRNAs were obtained from total RNA using FastTrack® MAG mRNA isolation kits (Invitrogen) allowing an oligo-dT based purification.

5 µg of mRNA were used to obtain cDNA. The synthesis of cDNA was performed using [α-^{32}P]dCTP to better calculate the efficiency of the reaction using SuperScript™ Plasmid System with Gateway™ Technology for cDNA Synthesis and Cloning (Invitrogen) as described below:

The major steps in constructing a directional cDNA library from an mRNA population using the SuperScript™ Plasmid System are summarized in figure below:



First strand synthesis

2 µl of Not I primer-adapter were mixed with 5µg (1µg/µl) of mRNA in a sterile 1.5-ml microcentrifuge tube. The mixture was added with following reagents:

4 µl of 5X First Strand Buffer
2 µl of 0.1 M DTT 2
1 µl of 10 mM dNTP Mix 1
1 µl of [α-32P]dCTP (1 µCi/µl)

5 µl of SuperScript™ II RT enzyme were used to retrotranscript mRNA in cDNA at 37°C for 1 h. 2 µl from the reaction were removed and diluted with 43 µl of 20 mM EDTA (pH 7,5) and 5 µl of yeast tRNA. This mixture was used to calculate first strand yield in a standard scintillant.

Second strand synthesis

The following reagents were added to the first strand reaction tube:

93µl of DEPC-treated water
30µl of 5X Second Strand Buffer
3µl of 10 mM dNTP Mix
1µl of *E. coli* DNA Ligase (10 units/µl)
4µl of *E. coli* DNA Polymerase I (10 units/µl)
1µl of *E. coli* RNase H (2 units/µl)

2 µl (10 units) of T4 DNA polymerase were used to synthesize the second strand.

The obtained cDNA was directionally cloned in the pEXPAD-502 yeast expression vector previously digested using two different restriction enzymes: SalI at 5' end and NotI at 3' end to enable the expression in yeast of virtually all the sequences cloned in the plasmid. The ligation reaction mixture was then introduced in ElectroMax DH10B™ E. coli cells by electroporation.

To determine the titre and the quality of the library several dilutions of the transformation mixture were plated on LB agar medium plus antibiotic. The remaining transformation mixture containing the primary library was added with glycerol and stored in -80°C until titre was determined. Once the titre was determined the primary library was amplified once on plates containing LB agar medium plus antibiotic and then stored in -80°C until use. From the titre plates 24 colonies were randomly picked and analyzed by enzymatic digestion using BsRGI enzyme that recognizes and cuts in *att* regions flanking the insert and the reaction was analyzed by electrophoresis.

Two-hybrid analysis

Two-hybrid analyses were performed with the GAL4 yeast two-hybrid system. Bait-coding TdRiSR cDNA were cloned in pDEST32 bait vector (Invitrogen) by Gateway recombination and transformed in yeast strain AH109. The library was introduced into *S. cerevisiae* strain AH109 carrying bait plasmid. Yeast cells were grown and transformed as previously described (Gietz and Woods 2001). Yeast colonies containing putative interactors were selected on medium containing SC without, leucine, tryptophane and histidine, supplemented with 5mM of 3AT. Colonies were picked after 6 days at 30°C. The prey-coding genes were identified by Sanger sequencing. Interaction was confirmed by a second round of two-hybrid analysis assay on SC medium

without adeninde, leucine, tryptophane, istidine supplemented with X-α-GAL (*MEL1*) and 5mM of 3AT. The cold treated library was screened according to the Gateway ProQuest Two hybrid system instructions (Invitrogen).

Nuclear localization assay

Nuclear localisation of TdRiSR-GFP was assessed with transient expression after particle bombardment in onion epidermis cells. Inner epidermal peels (2x2 cm) of commercial white onion were directly placed on agar plates containing Murashige and Skoog (MS) medium. Gold microcarriers (1 µm diameter) were prepared essentially as described by Dal Bosco et al. (2003). An amount of 35 µl of resuspended microcarrier (60 mg/ml ethanol) were mixed with 25 µg of TdRiSR-GFP plasmid or empty pMDC83 in negative control experiment. The microcarriers were delivered to the freshly transferred explants, using the Biolistic Particle Delivery System PDS-1000/He (Bio-Rad, Hercules, CA; Lemaux et al., 1996) according to the manufacturer's instructions.

A petri dish containing the plant tissue was placed 9 cm below the microcarrier launch assembly. The particles were fired using 1100-psi rupture discs (Bio-Rad) with a partial vacuum of 28 mm Hg. GFP was detected by reflected fluorescence system microscope, after recovering the transformed onion epidermis cells at +22°C for 18 h in the dark.

Protein expression and purification from *E. coli*

TdRiSR coding sequence was cloned in pMAL Gateway® converted vector for a fusion with MBP and cDNAs coding for JUBEL1, VIP2 and MAPK were

cloned in pGEX-2KT Gateway® converted vector for a fusion with GST. The vectors were provided by Dr.J.C. del Pozo. The constructs were transformed in BL21 Rosetta Z-competent *E.coli* cells according to manufactures instruction. Transformation mixtures wer plated on LB Agar plate plus Ampicillin and Chloranphenicol and incubated o.n. at 37°C.

A single colony was picked from each LB Agar plate and inoculated in LB liquid medium to prepare cell culture to induce the expression of the MBP or GST tagged proteins. The o.n. cultures were diluted 10 fold in a final volume of 100 ml incubated for 3 hours at 37°C with shaking and induced with IPTG. After 3 hours the cells were harvested by centrifugation and resuspended in PBS plus 0,1% of Tween20 with PMFS. The suspension was sonicated and incubated 48 hours at 4°C in presence of amilose or GSH-agar beads. After 48 hours the beads were recovered and washed 3 times with PBST plus PMSF to remove unbound proteins and then resuspended in PBST plus PMFS. MBP-TdRiSR recombinant protein was eluted from the beads by competition with 50mM of maltose. GST-interactors recombinant proteins were eluted from beads by competition with 50mM of reduced Glutathtione.

When TdRiSR E3 was expressed *in vitro* as His-tag fusion protein, Rabbit Reticulocyte Lysate System (Promega) was used according to manufactures instruction. For purification of His-tagged protein, MagZ™ Protein Purification System (Promega) was used.

Protein visualization by western blot

Purified protein were previously separated on a SDS-PAGE and blotted on PVDF membrane using semi dry electro transfer.

Semi dry electro transfer and immune detection

Proteins were transferred from SDS-PAGE to PVDF membrane methanol hydrated by semi dry electro transfer blotting system at a fixed current of 50mA during 1 hour in presence of running buffer.

PVDF membranes were analyzed following hybridization with antibodies using the dry method. To detect covalently attached biotinilated ubiquitin chains streptavidine-HRP were used, to detect MBP-TdRiSR fusion protein anti MBP antibody was used.

E3 ubiquitin ligase activity assays

The UBC8 (At5g41700) cDNA was cloned into the pGEX vector (Amersham, http://www.amersham.com). The recombinant GST-UBC8 was expressed in the BL21 bacterial strain in standard conditions, and was purified following the manufacture's instructions. Recombinant protein was released from beads with 50 mM of reduced glutathione in 20 mM Tris–HCl, pH 7, 20 mM NaCl, 5% of glycerol, and kept at) -80°C. MBP-TdRiSR was expressed and purified as described above. For the E3 ligase assay we mixed 2 µl of the MBP-TdRiSR fusion protein with 100 nM yeast E1, 0.1 µg of GST-UBC8, 0.2 mM DTT, 5 mM MgCl2 and 0.5 µg of biotynilated-ubiquitin, and 5 mM ATP. The reactions were incubated at 30°C for 45 min and then stopped by adding loading buffer with 100 mM DTT, and then incubated at 100°C for 10 min. for the control reaction sample buffer was added without DTT and boiling. In both cases, the proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, http://www.gehealthcare.com). These blots were blocked with 3% BSA in PBS-T (PBS1x with 0.1% of tween-20) for

1o minutes. Afterwards, the blot was incubated with Streptavidinhorseradish peroxidase (Sigma) at 1:50000 for 45 min to detect the Ub-biotin conjugated proteins. The blot was washed three times for 15 min each and then revealed with the ECL plus kit (Amersham).

When the assay was performed to evaluate the ubiquitination of the targets, the procedure was the same, only interactors were added to the mix and the ATP concentration was increased up to 10mM.

One-Dimensional Protein Fractionation

2,5 mg of 7 days old *Arabidopsis* seedlings, growth on MS agar plates, were incubated in liquid MS medium with and without 150mM NaCl for 6 hours at 200rpm and used for total proteins extraction in General Lysis Buffer plus PMSF and MG132 by sonication.

Total protein extracts were desalted using PD 10 cartridge (Amersham Bioscience) and eluted in start buffer (Beckman Coulter™).

The proteins from the two samples were fractionated independently by *pI* and hydrophobicity with a ProteomeLab PF 2D Protein Fractionation System (Beckman Coulter, Inc.). The first dimension separated the proteins by chromatofocusing, whereas the second is by reversed-phase chromatography. The first-dimension hardware consists of a manual injector, pump, pH monitor, and UV detector, whereas the second-dimension hardware consists of a binary pump and UV detector. Between the two dimensions, there is a combination fraction collector for the first dimension, which is used as the autosampler-injector for the second dimension. The chemistry components of this system include a chromatofocusing column, a high-performance reversed-phase column, and the start and eluent buffers for the first dimension. In this work only first dimension was performed.

In the first dimension, a chromatofocusing column was set at room temperature with a flow rate of 0.2 mL/min. This module had an injection loop of 2.0 mL. Before injection, the column was equilibrated with start buffer (pH 8.5) for 130 min. The pH electrode was calibrated with pH 4,7 and 10 buffers before the start of the run. The first-dimension separation was started with the sample injection; in the first 20 min, start buffer was pumped through the column to elute proteins with *pI* value above 8.5. After 20 min, the pH gradient from 8.5 to 4.0 was started by changing the elution solvent to eluent buffer (pH 4.0). After the pH gradient (115 min), a 1M sodium chloride solution was used to remove proteins with *pI* values below 4.0 from the column. The final step was to rinse the column with water.

Membrane ubiquitination assay

The protein fraction were subsequently transferred to a PVDF membrane by dot-blot using the BIO-DOT™ apparatus (BIORAD) and incubated o.n. with the BSA blocking solution. Blocked membrane was incubated with 1ml of ubiquitination mix containing 500ng of E1, 500ng of E2 and 300ng of MBP-E3 or MBP alone in presence of biotinilated ubiquitin during 3 hours at RT. Membrane was incubated with streptavidine-HRP in presence of 2,5% of BSA during 1 hour and revealed following an incubation of 5 minutes with ECL plus (Amersham).

RESULTS

TdRiSR SHOWS E3 UBIQUITIN LIGASE ACTIVITY *IN VITRO*

TdRiSR coding sequence (CDS) encodes a 429 amino acid protein containing a C3H2C3 type RING finger domain (aa 281-334, Figure 9) with a predicted molecular weight of 46 kDa (Mastrangelo et al., 2005). The analysis of the aminoacidic sequence of TdRiSR by the on line tool MYR-Predictor (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm) identified a myristoylation signal at its N-terminal region (Fig. 9). To identify potential phosphorylation sites, the *Arabidopsis* homologous gene *At4g39140* was searched in the using PhosPhAT3.0 (http://phosphat.mpimp-golm.mpg.de/statistics.html). A predicted phosphorylation site confirmed by experimental data is present in position 400 on the peptide HF(pS)FGSR. As this region is highly conserved between At4g39240 and TdRiSR, the wheat protein could be phosphorylated at the same Serine residue.

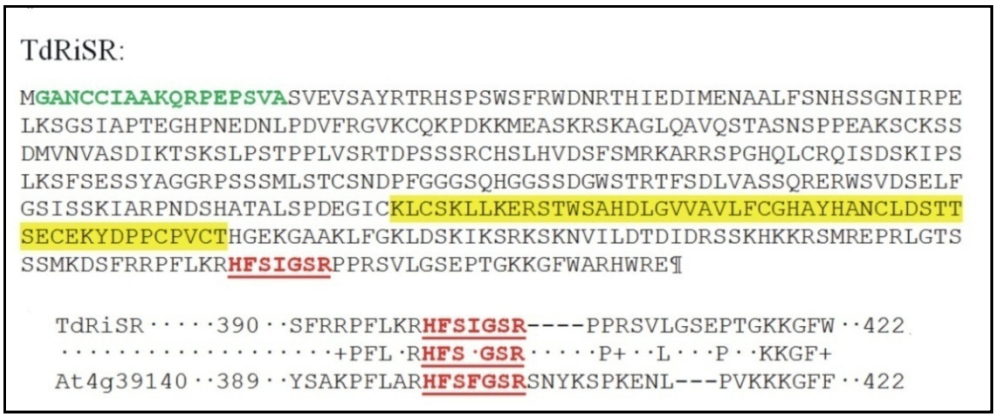


Figure 9: Aminoacidic sequence of TdRiSR deduced from sequencing the cloned gene. Yellow highlighted region corresponds to RING-finger domain, bold red underlined region corresponds to phosphorylation site, bold green region is the N-Myristoylation signal. The alignment between TdRiSR and At4g39140 is shown only for the region containing the phosphorylation site.

The RING-finger is protein–protein interaction domain involved in protein ubiquitination (Matsuda et al., 2001), being typical of many E3 ligase enzymes. To verify the ubiquitin ligase activity of the RING finger protein TdRiSR, an ubiquitination assay has been carried out. The His-tagged TdRiSR fusion protein was expressed in an eukaryote system (Rabbit reticulocite, Promega) to allow the expression of the protein in presence of all cellular components thus allowing posttranslational modification, if needed. Although the amount of protein that was recovered was low there was sufficient material to run an ubiquitination assay experiment. The work was performed in a test tube where TdRiSR from wheat, E1 from yeast and E2 from wheat were mixed with cold ubiquitin as previously described by Stone et al., (2005). Creatine kinase and phosphocreatine were also added in the reaction mixture to maintain ATP levels. The reaction was analyzed by western blot with ubiquitin specific antibody (Fig. 10).

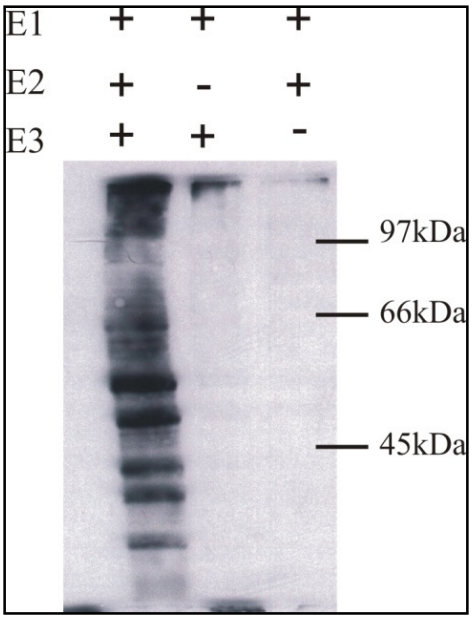


Figure 10: TdRiSR ubiquitination assay Polyubiquitinated protein were identified with anti ubiquitin antibody. E3 is the TdRiSR fused to His tag obtained from in vitro translation reaction.

The results indicate that polyubiquitin chains are detectable only when TdRiSR E3 is present together with E1 and E2.

Given that the protein level expressed using the previous system was not enough to perform further experiment, an alternative expression system was employed to obtain a higher amount of purified protein. For this purpose, TdRiSR was expressed in *Escherichia coli* as a fusion with maltose-binding protein (MBP), a 42 kDa tag for purification through its binding to amilose.

The full length CDS was subcloned in the pMAL vector using the GATEWAY® technology. The pMAL vector allows to produce a N-terminal MBP construct under the control of a T7 promoter whose induction is IPTG mediated. This system provides an useful tool to express and purify recombinant proteins by the use of affinity chromatography. BL21 Rosetta *E. coli* cells were used for protein expression, as this strain contains an additional plasmid coding for tRNAs that are rare in a prokaryotic system thus improving the translation efficiency of full length recombinant eukaryotic proteins.

After 3h expression, TdRiSR fused to MBP was purified through amilose agar beads. To verify the amount of MBP-TdRiSR fusion protein bound to the amilose beads, the beads were treated with Laemmli buffer, eluted proteins analysed by SDS-PAGE and Blu Comassie staining (Fig. 11). As shown in Figure 11 high amount of a 88KDa protein which corresponds to the expected molecular weight of MBP-TdRiSR fusion protein was purified. However additional bands were also present probably due to a premature stop during translation in *E. coli*. Indeed, given that the MBP tag is fused to the N-terminal end of TdRiSR protein, all truncated species can bind amilose and co-purify with full length MBP-TdRiSR. However, the latter constitutes the main product.

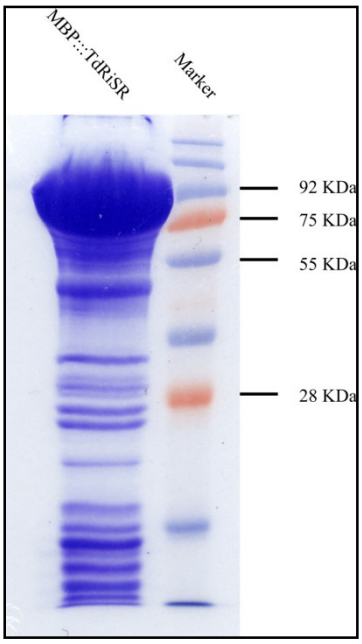


Figure 11: Purification of the MBP-TdRiSR fusion proteins. MBP-TdRiSR fusion proteins bound to amilose beads. Analysed by SDS-PAGE and Blue Comassie staining.

MBP-TdRiSR fusion proteins were released from the beads by competition with 25mM maltose and further analyzed by immunoblotting with anti MBP antibody (Fig. 12). Again additional bands below that one corresponding to the full length fusion protein are visible due to the presence of MBP-containing truncated forms of the recombinant protein.

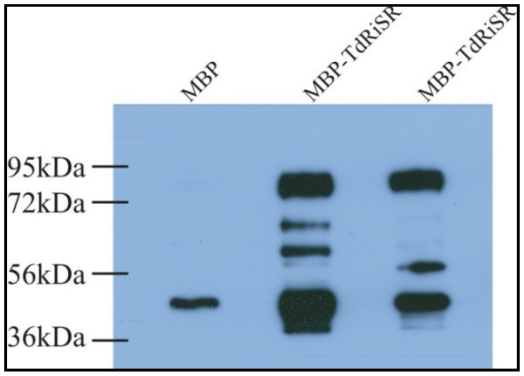


Figure 12: Western blot on purified MBP-TdRISR with anti-MBP immunoglobulins. From left to right: MBP alone as control for antibody, MBP-TdRiSR Fusion protein expressed in BL21 Rosetta strain, MBP-TdRiSR Fusion protein expressed in DH5α strain.

To perform the ubiquitination assay experiment, 500 ng of the eluted protein was mixed with human ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme GST-UBC8 (E2), and ubiquitin (biotinylated ubiquitin). Thus the entire ubiquitination pathway is assembled *in vitro*, together with control assays represented by reaction mixes without E3 or E2 enzymes (Fig. 13). As no actual targets are present, the activity of the pathway is expected to cause ubiquitination of E1 or E2 or self-ubiquitination of E3. Reactions were performed as reported by Jurado et al., (2008). After an incubation time of 30 minutes at 30°C, half volume of each reaction was collected and treated with Laemmli buffer in presence of a reducing agent and boiled. The second half of each reaction was treated with Laemmli buffer, without reducing agent nor boiling. All samples were then analysed by SDS-PAGE and immunoblotting with streptavidin to recognise biotinylated ubiquitin. The non treated samples work as positive controls of the ubiquitination system. Indeed, during ubiquitination E1 and E2 bind ubiquitin via a thioester bond which can be broken by boiling the reaction in presence of a reducing agent, while ubiquitin molecules bound in a covalent manner can not. By comparing boiled and unboiled samples it is possible to determine if the reaction worked and to exclude a failure in the system in case of negative results.

In the presence of ubiquitin, E1, E2, and purified MBP-TdRiSR, polyubiquitinated proteins were formed (Fig.13B). This ubiquitination activity depended on the presence of MBP-TdRiSR and E1, E2 as well (Fig. 13B). Predicted molecular weights of the E1 and GST-E2 are around 115kDa and 45kDa, respectively. According to the molecular weight of the polyubiquitinated proteins, targets of the covalent attachment of ubiquitin molecules in this assay are mainly E2 proteins, and, to a lesser extent, E1 proteins. The results suggest that TdRiSR possesses E3 ligase activity as hypothesized. Moreover, as E1 looks covalently ubiquitinated even in absence of E2, TdRiSR protein can accomplish ubqiuitination using the E1 as a target.

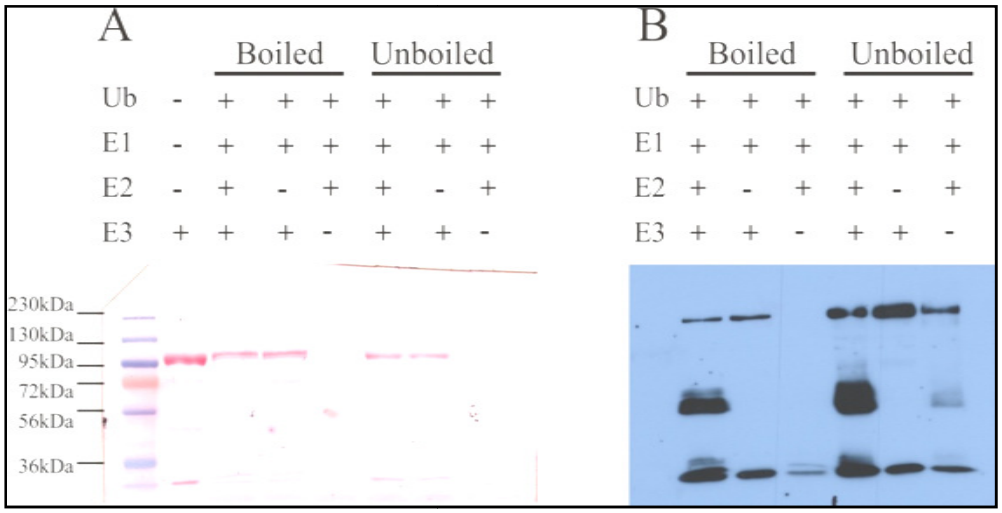


Figure 13: Ubiquitination assay for TdRiSR. A: PVDF membrane stained with Ponceau Red, visible bands correspond to MBP-TdRiSR. B: western blot of the ubiquitination reaction; biotinilated ubiquitin was used, and the western was performed using streptavidine.

The different amount in polyubiquitin chains between the two experiments can be attributable to a different activity of TdRiSR due to the different reaction conditions employed. Notwithstanding, the two ubiquitination assays indicate that TdRiSR acts as E3 ubiquitin ligase and suggest that the activity is strongly affected by availability of ATP.

TdRiSR IS NUCLEAR LOCALISED

When the TdRiSR amino acid sequence was scanned for subcellular localization signals using WoLF PSORT (http://psort.ims.u-tokyo.ac.jp/), a nuclear localization was predicted. To verify the prediction, the *TdRiSR* gene was subcloned in the pMDC83 Gateway vector for *in planta* expression of the GFP-TdRiSR fusion protein under the control of the CaMV 35S promoter. Onion epidermal tissues were chosen for transient transformation by particle bombardment, because of their large size and absence of chlorophyll. As

previously reported by Marè et al. (2004), besides the construct (Fig. 14C and D) the empty vector encoding GFP only was transformed as a negative control (Fig.11A and B). Green fluorescence was observed after a 18-22h incubation at 22°C. Green fluorescence between 460-490 nm (Fig. 14A,14C) and the corresponding images under white light (Fig. 14B,14D) are shown at the same exposition time.

Onion cells transformed with a plasmid expressing GFP alone showed fluorescence throughout the cell, because of the free diffusion of the small size GFP protein. In contrast, fluorescence was detected exclusively into the nucleus of cells transformed with the plasmid expressing the TdRiSR-GFP fusion protein, indicating the nuclear localization of TdRiSR protein.

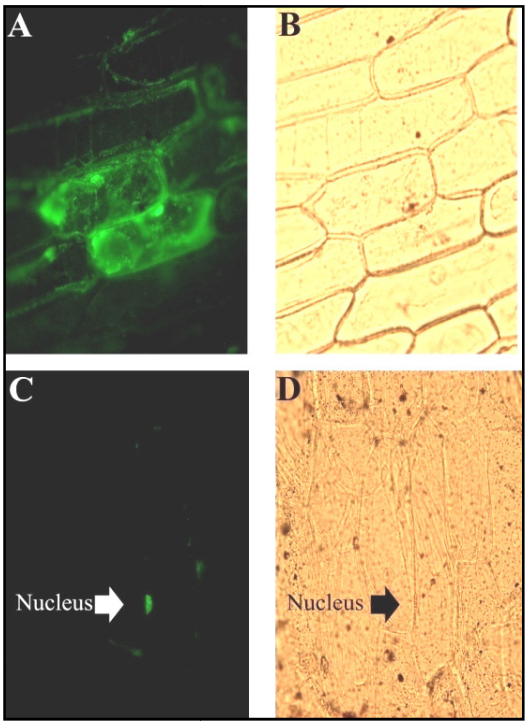


Figure 14: Nuclear localization of the TdRiSR-GFP fusion protein by means of transient expression assays of GFP fluorescence in transformed onion cells. Onion epidermal cells were transformed by particle bombardment with a construct encoding the TdRiSR-GFP fusion protein (C, D) or with a construct encoding GFP only (A, B). Green fluorescence excited by wavelength between 460 and 490 nm (A, C) and the corresponding images (B, D) under white illumination are shown at the same exposition time.

DEVELOPMENT OF A WHEAT cDNA EXPRESSION LIBRARY

Identifying protein interactors of a target protein allows to place it within the cellular networks in order to understand its role into the cell. The yeast two-hybrid system was therefore applied to identify protein interactors of TdRiSR. They can be both proteins ubiquitinated by TdRiSR and regulators of TdRiSR activity. For this purpose a wheat cDNA expression library was developed in yeast A cDNA library is an array of DNA copies of an mRNA population that are propagated in a cloning vector and usually maintained in *E. coli*. A high quality cDNA library has three key characteristics:

1. It is large enough to contain representatives of all sequences of interest, including those derived from low-abundant mRNAs.
2. It includes a minimal number of clones that contain small (often defined arbitrarily as ≤500 bp) cDNA inserts.
3. It is composed of cDNA inserts that are near full-length copies of the mRNAs from which they were derived.

In this work 5µg of mRNA, obtained from 7 days old wheat seedlings exposed 6h at 3°C, were obtained by purification with FastTrack® MAG mRNA Isolation Kits (invitrogen) and used in a retrotranscritpion reaction. The corresponding cDNA was cloned in pEXPAD-502 yeast expression vector. The obtained *E coli* library was characterised to establish its quality features: the cDNA expression library contained 10^6 independent clones and the estimated average insert size was 2000bp (Fig. 15), the percentage of empty vectors was 0% suggesting that the library really contains 10^6 independents clones without false-positives. After the first amplification the titre of the library was 5,5 x 10^{10} Cfu/ml (colony forming units/ml).

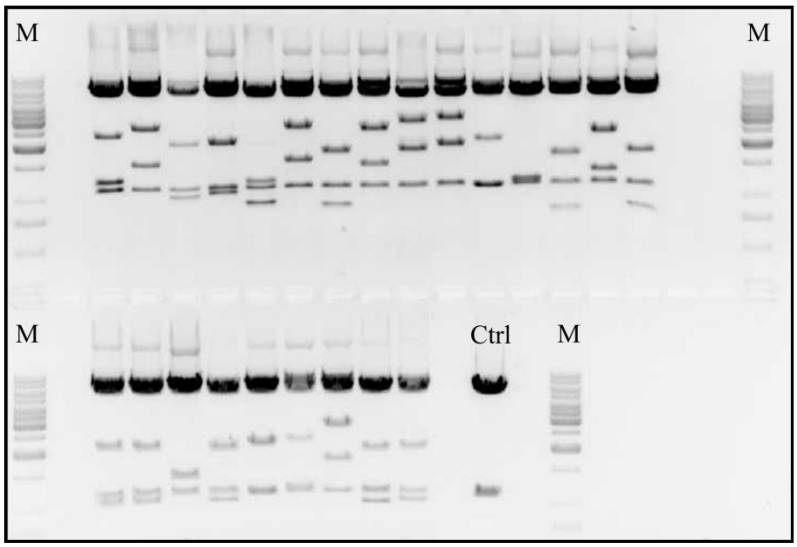


Figure 15: electrophoresis analysis of expression library. 24 colonies were randomly picked and analyzed by enzymatic digestion using BsRGI enzyme that recognizes and cut in att regions flanking the insert. The selected colonies contain 106 independent clones, estimated average insert size is 2000bp, the percentage of empty vectors is 0% suggesting that the library really contains 106 independents clones without false-positives (Ctrl=control [pEXPAD-502 empty vector], M=Marker).

TWO-HYBRID SCREENING

The system is based on the topology of the *GAL4* transcription factor, a 881 amino acids protein from *S. cerevisiae* containing a DNA-Binding Domain (DB, aa 1-147) and a transcription Activation Domain (AD, aa 771-881), (Young, 1998). Three elements are characteristic of this system: a plasmid vector, reported as bait vector, to express your favourite gene as a fusion protein with the *GAL4 DB*, a plasmid vector, reported as pray vector, to express the gene supposed to interact with the bait or an expression library as a fusion protein with the *GAL4 AD*, a reporter gene (or several) whose promoter contains the binding sequence for *GAL4 DB* to detect protein-protein interactions.

To perform the two-hybrid screening the coding sequence of TdRiSR gene was cloned in the pDEST32 GATEWAY® vector to create bait construct. Yeast

strain AH109 was transformed with bait construct as previously described (Gietz and Woods 2001).

The HIS3 reporter gene, used in this system, shows a basal expression level which is inhibited in a dose dependent manner by 3AT (3-amino-1,2,4-triazole). To avoid false positives during the screening, that is response of the reporter gene due to bait autoactivation, basal concentration level of 3AT that inhibits cells growth was determined. As shown in Figure 16 the threshold level of 3AT that inhibits yeast cells growth carrying bait plasmid and empty pray vector is 5mM.

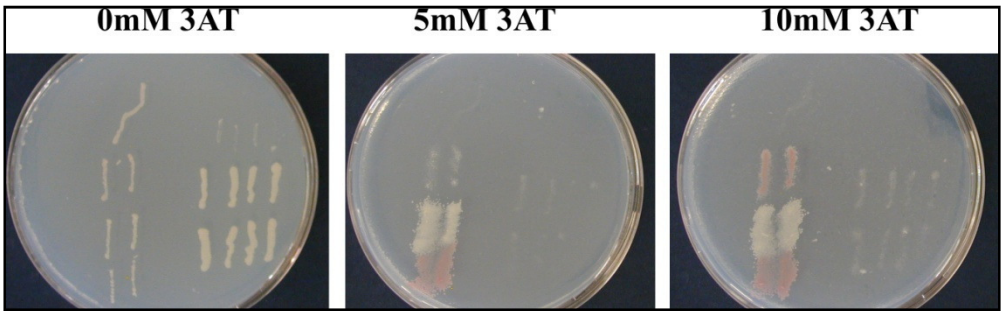


Figure 16: Auto activation assay. The threshold level of 3AT that inhibits yeast cells growth carrying bait plasmid and empty pray vector is 5mM.

To develop the yeast expression library, yeast strain AH109 carrying bait plasmid was transformed with a mega plasmid preparation of the *E coli* library, as previously described (Gietz and Woods 2001). The transformation was plated on medium on SC-Leu-Trp-His for selection of trasformants for both vectors (Leu and Trp auxotrophy) and for selection of interacting proteins (His), added of 5mM 3AT to avoid selection of auto activating clones. The efficiency of the transformation reaction was calculated by plating dilution of transformation on a SC-Leu-Trp medium that allows selection for transformants only. In this work $8x10^6$ clones were screened in yeast resulting in 600 colonies carrying putative interactions.

Given the high number of colonies resulting from the screening and due to clone redundancy, a colony sorting step was carried out. After plasmid preparation, inserts were PCR amplified using prey vector specific primers, amplicons digested by a four cutter enzyme and analyzed by electrophoresis (Fig. 17).

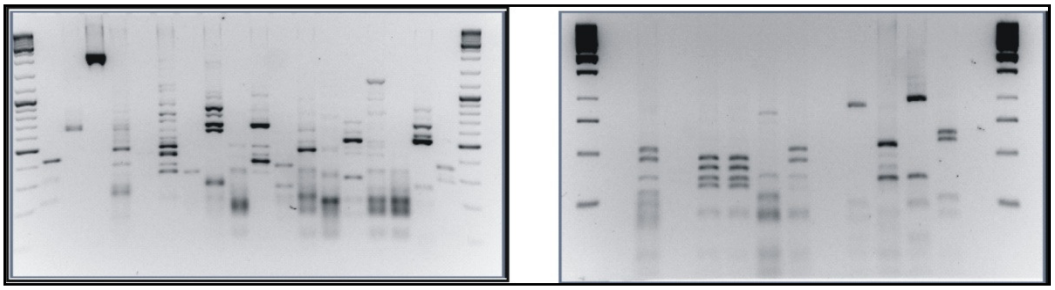


Figure 17: colony sorting of potential interaction to reduce number of redundant clones. The colonies were incubated in 96 deep well plates in liquid SC-Leu-Trp medium o.n. at 30°C. Cell cultures were used for automated plasmid preparation and plasmid DNA from each sample was amplified by PCR using primers specifics for the prey vector and the amplicons digested using a 4 cutter enzyme and analyzed by electrophoresis. The image shows only representatives gels.

At the same time clones were sequenced, data obtained used for clone identification by sequence similarity throughout NCBI and TIGR database using Blast software. 21 different wheat TC (Tentative Consensus) were identified 16 of which with a predicted function based on annotation. According with previous results on TdRiSR nuclear localization, clones whose product is predicted having a subcellular localization different from nuclear were discarded, as well as less abundant clones (found less than 3 times). After this selection, 8 clones were kept and subjected to retransform assay.

In table 2 the results obtained from Blast search and the sequences for which the retransform assay was carried out are shown.

TC or EST	Protein tentative annotation	Frequency in 2HY	Putative subcellular localization	Confirmed in retrasform assay
wheat|TC316365	Glycoprotein protein (MAM33 superfamily)	84	Mitochondrion	X
wheat|TC289081	Homologous to *Avena f.* VIP2 protein	34	Nucleus	X
wheat|CV778274	Dihydrodipicolinate reductase	13	Chloroplast	-
wheat|TC282860	WNK protein kinase	12	Chloroplast/ Nucleus	X
wheat|TC288300	holocarboxylase synthetase	9	Mitochondrion/Cytosol	-
wheat|TC344139	Glycosil transferase – Immediate early protein ICP0 like	8	Chloroplast	X
wheat|TC314363	Hypothetical protein	7	Chloroplast	-
wheat|TC298279	Smr (Small MutS Related) domain-containing protein-like	5	cytosol	X
CA684684	Homologous to *Hordeum v*. JUBEL1	4	Nucleus	X
wheat|TC320440	S-adenosylmethionine decarboxylase	4	Cytosol/Chloroplast	-
wheat|TC288467	Calcium-dependent protein kinase CPK1	3	Nucleus/Cytosol	X
wheat|TC310251	GTP cyclohydrolase II activity	3	Chloroplast	-

wheat|TC29 8963	Aminomethyltransferase	2	Chloroplast	-
wheat|CJ956 110	Heavy meromyosin-like	2	Nucleus/cytosol	-
CV761439	Hypothetical protein	3	-	X
wheat|TC33 4904	-	2	Cytosol	-
wheat|TC31 5324	-	2	Cytosol	-
wheat|TC28 4926	Cysteine synthase	1	Chloroplast	-
wheat|TC27 7570	Cold acclimation protein WCOR518	1	Chloroplats	-
wheat|TC30 7771	Leucine zipper protein zip1	1	Nucleus	-
wheat|TC29 6448	Hypothetical protein	1	Chloropast	-

Table 2: In the table are listed al TC identified in the library screening with predicted function, subcellular localization, number of presences in the screening and is they are tested for retransform assay or not.

Retransform assay was performed to confirm protein-protein interaction in yeast. Putative interactors were transformed in yeast strain Y187 and the bait construct carrying TdRiSR CDS in AH109 strain as previously reported (Gietz and Woods 2001). Diploid cells carrying both plasmids were obtained by mating procedure. Diploids cells were plated on the high stringency medium SC-Leu-Trp-His-Ade plus 3AT and plus X-α-GAL for colorimetric assay. The stringency of the medium used for retransform assay is higher than that used for screening thus avoiding false positives coming from the initial experiment.

As shown in figure 18, 3 out of 8 selected clones were able to grow on high stringency medium, nevertheless in presence of X-α-GAL colonies turned in blue indicating the presence of interaction. On the contrary, no growth was visible for control transformation (cells carrying prey constructs and empty bait vectors) (Fig. 18E, F, G). The small amount of cells visible for auto activation was due to a large starting amount of cells used for the initial inoculation, rather than actual growth (Fig. 18D).

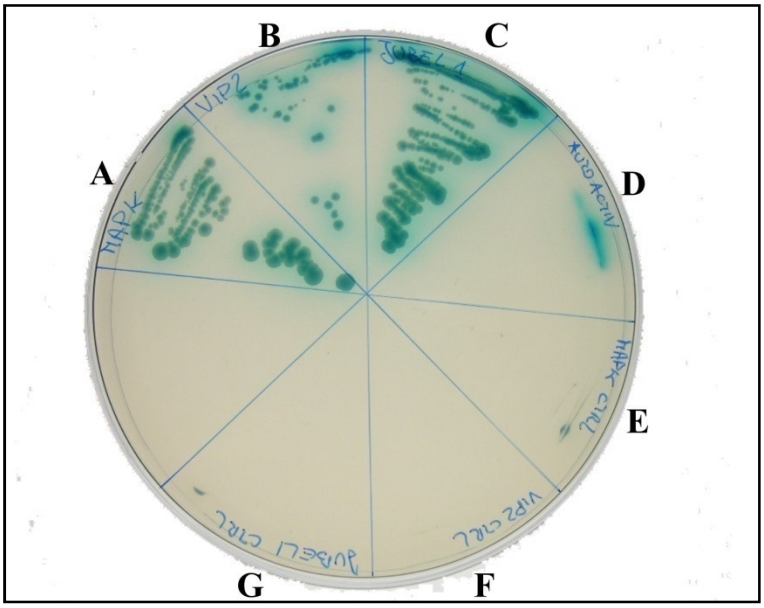


Figure 18: retransform assay on selected clones. Three clones are able to activate the reporter genes indicating the interaction with TdRiSR protein (A, B, C). Control transformationation (D, E, F, G) are not able to grow on medium selective for interaction. Only genes that were able to grow are showed in this image. A = DB-TdRiSR + AD-MAPK, B = DB-TdRiSR + AD-VIP2, C = DB-TdRiSR + AD-JUBEL1, D = DB-TdRiSR + AD, E = DB + AD-MAPK, F = DB+ AD-MAPK, G = DB+ AD-MAPK.

To conclude, by two-hybrid system three gene clones encoding proteins that interact with TdRiSR in yeast have been identified: a protein homolog to barley JUBEL1 transcription factor (Muller et al., 2001), a protein homolog to *Avena fatua* VIP2(VP1 interacting protein) gene (Jones et al., 2000) and a durum wheat MAPK (Mitogen – Aactivated Protein KInase) homologous to members of the

Arabidopsis WNK (With No Lysine [K]) family of MAP kinases (Wang et al., 2007).

JUBEL1 is an Homeodomain protein belonging to TALE (Three Amino acid Loop Extension) superfamily that interacts *in vitro* with the class I KNOX protein BKN3 (Muller et al., 2001); VIP2 is a RING-finger domain (H2 type RING-finger domain, C-X_2-C-X_{9-39}-C-X_{1-3}-H-X_{2-3}-H-X_2-C-X_{1-48}-C-X_2-C) containing protein that interacts *in vitro* with Maize VIVIPAROUS 1 (Jones et al., 2000). Regards the MAPK no data are available but is known that *Arabidopsis* WNK Kinases protein are involved in regulating flowering time and photoperiod pathway (Wang et al., 2007). It is likely that these proteins interact with TdRiSR to be ubiquitinated or alternatively to regulate its activity, above all in case of VIP2 and the MAPK protein. None of the gene insert carried by interacting clones recovered by two-hybrid system were full-length due to the premature interruption of retrotranscription reaction. Below are showed aminoacidc sequences of three interactors.

JUBEL1:

GVLYFNRQQQQQQQQQAASVQQLPMALHGQVGSMGQQLHVGYGPAGVAGVLRNSKYTRAAQELLDEFCSVGRGQTIKGGG
RGGSSSNPNASKGGPSSSGAAQSPSSASKEPPQLSPADRFEQQRKKAKLISMLDEVDRRYNHYCDQMQMVVNFFDSVMGF
GAATPYTALAQKAMSRHFRCLKDAIAAQLRHTCELLGEKDAGTSSGLTKGETPRLRAIDQSLRQQRAFHHMGMMEQEAWR
PQRGLPERSVSILRSWLFEHFLHPYPSDADKHLLARQTGLSRNQVSNWFINARVRLWKPMIEEMYQQETKELEGSSAAAG
GGGGVGGPESGNDPSGADDLHSPTTTGSQQQLVIHHGGGRYGQQEHGMSGVHPHKLDPGAGPSVADAAFVGLDPAELLGG
DAHVGAADDLYGRFEPGVRMRYGPATTGAVSGDVSLTLGLQHAGAGNQGPDGSGRFSLRDYNGC

MAPK:

AARNSDNGYVETDPTGRYGRFDELLGKGAMKSVYRGFDEVRGVEVAWNQANLADVLRTPDALQRMYSEVHLLSTLRHDAI
IAFHASWVSVSSPSPRGGCTGGTPRRTFNFITELFSSGTLRAYRLRYPRVSLRAVRGWARQILRGLAYLHAHDPPVIHRD
LKCDNVFVNGHQGTVKIGDLGLAAVLRGAQAAHSVIGTPEFMAPEMYDEDYDELVDVYSFGMCMLEMLTVEYPYAECSNP
AQIYKKVTSGKLPDAFYRVDDADARRFIGRCLVPASHRPSAQELLLDRFLSTQDTTMTLSPPPLLPALPTSGDRKDNPEE
AEPVAARTDMTITGKLNTDDDTIFLKVQIVDEAGHSRNIYFPFDIAGDTATEVAREMVKELDITDRDPSEIAAMIEQEIT
RLVPDWVGGGCDDQQEYYTYADNDDNEEQPPFYYLSSSPTSSNGSHCGTGPTTSGGGYAGWFQDYAVSSDDDETSSTRSA
LHYSSEEAQPEEKPGVSKTGQVKATRFGPGDSGTAGHDVSSSRAGRPRHHRGSPDAGGDEGRP

VIP2:

GEEEPLEVEEGAGGKEEKAAVSCSICLDAVVAASAERSTARLQCGHEFHLDCIGSAFNAKGVMQCPNCRKIEKGNWLYAN
GSRPSHDINMDEWAHEEDLYDVSYSEMPFRFHWCPFGRLAQLPSFFEEGESPPPVTFHDFMGQHVFPENLSVSAAPGAHP
CPYVAYLHPLPSLASSSSSHVPERTMDGSAYHDHWNHLAGPSDGRPLQTVQPTDFHHNHWAHLPHSYVQSNGNNGVTEQP
GVPFGSMRAARVDGDSQRRGSVVSPSYFSNGSGSRSRAPNVPPLVPQFMRAHGNINEQYTQSSSSSLFAGAHRSGGMRPA
PPPPQPENPTFCLFPPGSSGHSSMDTDEAGGSRVYAWERDRFAPYPLMPVDCETRGWSSQQSHGASESTPAPAPRRLFGQ
WIGLGRSSPENRSPEGSSYRQMHSPWM

Figure 19: aminoacid sequences of the three interactors. For JUBEL1 sequence the POX domain, a domain exclusively present in plants and associated with homeobox function (Doerks et al., 2002) is highlighted in Grey, the Homeobox_2 domain is highlighted in Green and the Homeobox_1 domain is

highlighted in light blue; for the MAPK the protein kinase domain is highlighted in violet and for VIP2 sequence the RING-finger domain is highlighted in yellow and cisteines and histidines are underlined in bold.

TdVIP2 SHOWS E3 UBIQUITINATION ACTIVITY

To test if the interactors identified by two-hybrid system are really target of TdRiSR ubiqutin ligase, an ubiquitination assay including interactors was carried out. For this purpose, the three interactors were expressed in *E. coli* as a fusion with Gluthatione S-Transferase (GST), a 27 kDa tag for purification through its binding to GSH-beads. The available CDSs of the 3 interactors were subcloned in the pGEX-2TK vector using the GATEWAY® technology. The pGEX-2TK vector allows to produce a N-terminal GST construct under the control of a T7 promoter whose induction is IPTG mediated. This system provides an useful tool to express and purify recombinant proteins by the use of maltose affinity chromatography. The three interactors were induced and purified as did for TdRiSR protein, although in this case recombinant proteins were realised from the beads by competition with 50mM of reduced Gluthatione. Purified protein were visualized by Blue Coomassie staining (Fig. 20). Several additional bands below the main band of each interactor are visible due to the presence of truncated forms of recombinant protein carrying GST tag.

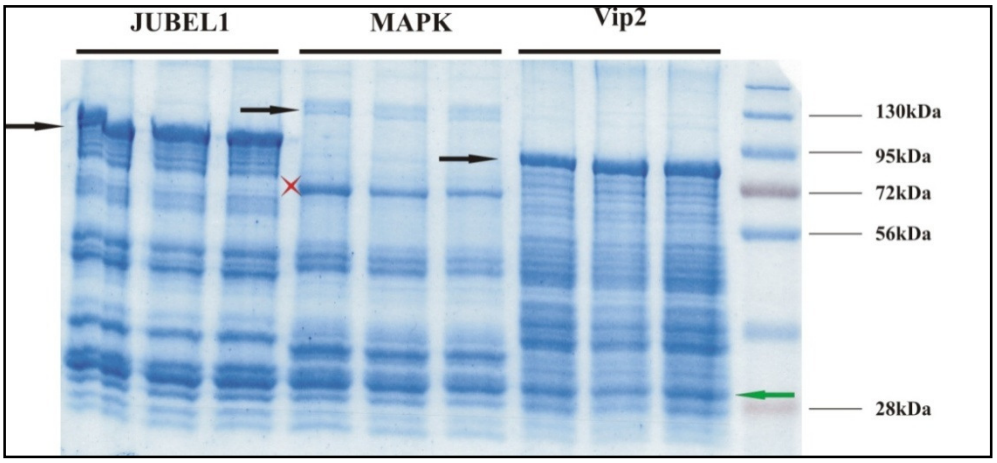


Figure 20: SDS-PAGEe electrophoresis and Blue Comassie staining for the visualization of GST-interactors fusion proteins bound to GSH beads. Black arrows indicate the band corresponding to fusion proteins, green arrow indicates the band corresponding to GST peptide, red star indicate the most abundant protein specie for MAPK although is not the full length.

The ubiquitination experiment was performed essentially as that for TdRiSR but including the interactors in the reaction mixture. As shown in Figure 21, a signal was present in the unboiled samples indicating that biotinylated ubiquitin was bound to some proteins; however, when samples were boiled the signal disappears indicating that the ubiquitin was not bound in a covalent manner. Surprisingly these results indicate that the interactors are not target of TdRiSR - mediated ubiquitination.

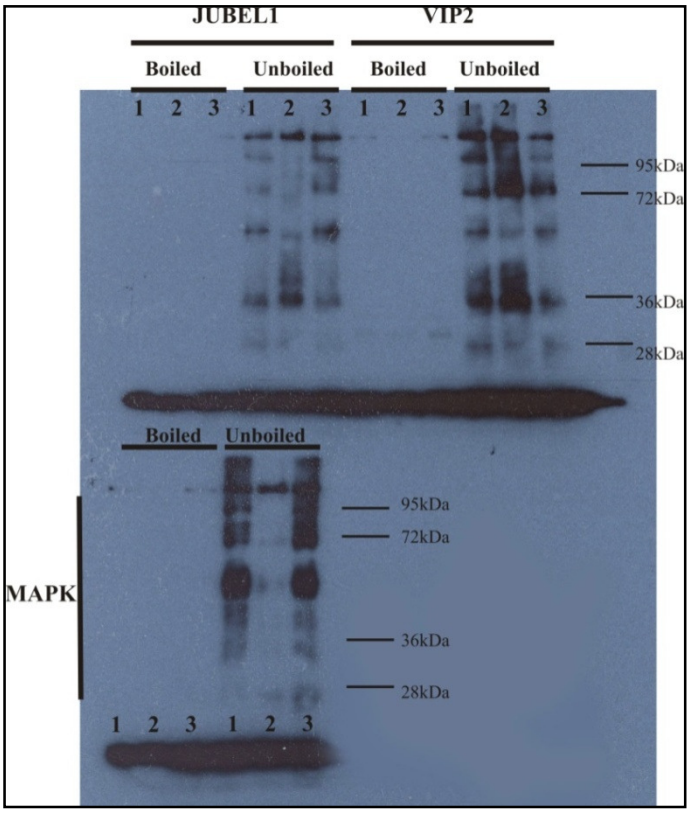


Figure 21: ubiquitination assay using interactors as targets. Biotinilated ubiquitin was used for this assay and revealed with streptavidine-HRP. Basic ubiquitination mixture is E1+E2+TdRiSR (as E3); for each target tested the order is the following: Lane 1= Basic mixture – E2; Lane 2= Basic mixture – E3; Lane=3 Basic mixture +interactor.

Three reasons can explain these results:

- the three interactors are regulators of TdRiSR rather than TdRiSR targets
- as TdRiSR sequence contains a phosphorylation signal, phosphorylation could be a prerequisite for TdRiSR functionality
- TdRiSR expressed in *E coil* is low active because of lacking of some other post translational modifications or a incomplete folding due to the large MBP tag.

On the basis of this observation new ubiquitination assays were done for JUBEL1 and VIP2, increasing the amount of ATP in the reaction mixture up to 10mM and including MAPK (Figure 22).

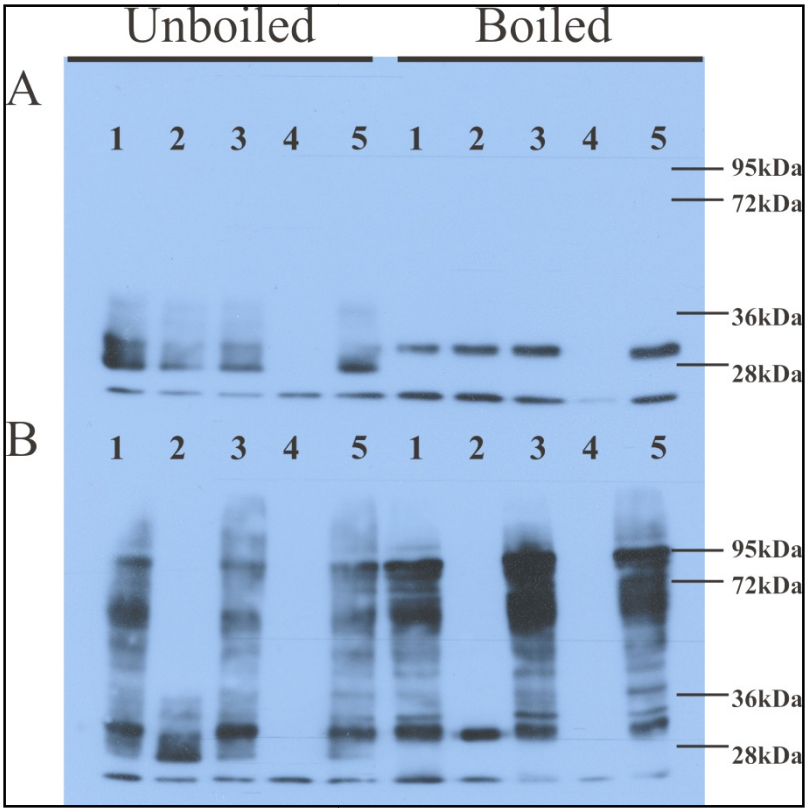


Figure 22: ubiquitination assay using interactors as targets. Biotinilated ubiquitin was used for this assay and revealed with streptavidine-HRP. Basic ubiquitination mixture is E1+E2+TdRiSR (as E3)+MAPK; for each target tested the order is the following: Lane 1= Basic mixture+VIP2; Lane 2= Basic mixture; Lane 3= Basic mixture +interactor-TdRiSR; Lane 4=. Basic mixture +interactor-E2; Lane 5= Basic mixture +interactor-MAPK. The upper side of the image refers to JUBEL1 while the lower side refers to VIP2.

When the experiment was performed using JUBEL1 as target no signal was visualized (Figure 22A), but when VIP2 was used a strong signal of polyubiquitination was observed (lane 1, 3, and 4, Fig 22B).

The signal corresponding to polyubiquitinated proteins is present when the E1, the E2 and VIP2 are presents in the mixture reaction at the same time.

Including or omitting the TdRiSR or MAPK proteins does not influences the strength of the signal while if VIP2 or the E2 are omitted (lane 4 and 2 respectively) the signal disappear. These results indicate that presence of polyubiquitinated proteins strongly depends from the presence of the E2 together with VIP2 indicating this last as the protein possessing E3 ubiquitin ligase activity.

SETTING UP OF A NEW METHOD TO IDENTIFY E3 TARGET PROTEINS ON LARGE SCALE

During the Ph.D. the candidate was awarded with an EMBO fellowship to spend 6 months in the Dr. del Pozo´s lab at the CBGP-INIA (Centro de Biotecnologia y Genomica de Plantas – Instituo Nacional de Investigation y Tecnologia Agraria y Alimentaria) in Madrid. During this period the candidate was involved in a project aimed to develop a new system to identify E3 targets in *Arabidopsis* plants on large scale. Preliminary results of the work are showed.

Several E3s that showed a clear induction by salinity stress were identified by Dr. del Pozo's group. Two of them were selected for further analysis since they are also regulated by drought based on *in silico* analyses using the geneinvestigator program. The cDNAs of the selected putative E3s were cloned into the pDONOR221 Gateway compatible vector and then transferred to the pMAL vector for their expression in bacteria as recombinant protein fused to MBP. The recombinant proteins were analyzed for E3 ubiquitin ligase as described by Stone et al. (2005). To set-up the method only one E3 enzyme was used.

In order to identify the ubiquitin targets of this E3, first the *Arabidopsis* proteome of salt treated plants was fractionated using the two-dimensional

liquid chromatography system PF2D. This equipment is able to separate a total extract protein by their isoelectric point (*pI*) and by hydrophobic interactions through two sequential liquid chromatography columns (http://www.beckmancoulter.com/products/instrument/protein/proteomelab_pf2d_dcr.asp). Separated proteins are individually recovered in a set of multi-well plates. Then ubiquitin assays are accomplished in each well, that is on every separated protein, by adding the target E3 and the other components of the ubiquitination pathway in order to test all proteins as specific target of the E3 activity.

Arabidopsis seedlings growth in a MS liquid medium for 7 days and then treated with or without salt were used to extract total protein in Urea-containing buffer and equilibrated for loading in the PF2D equipment following the manufacter´s instructions. In this preliminary set up of the method, the proteome was one-dimension fractionated in 32 fractions depending on their Isolectric Point (*pI*) and proteins were collected in 96-well plates.

Afterwards, the collected fractions were spotted, in duplicated, onto PVDF membrane using a dot-blot system. These membranes were incubated during 24 hours to allow the re-naturalization of the proteins. At this stage, membranes were incubated with yeast E1 (Biomol), GST-E2 (UBC8) and an ubiquitination solution that contains biotinylated-ubiquitin and the E3 (Assay, Fig 23) or MBP only for Control reaction (Control, Fig 23). After 3 hours reaction at RT, membranes were washed with PBS-T to eliminate reaction components, including polyubiquitinated E1 and E2 enzymes. Them membranes were incubated with Streptavidin-HRP (1:50000) during 1 hour at RT for hybridization with Biotine. The membranes were analyzed by chemiluminescence using the immobilion system (Milipore; Figure23).

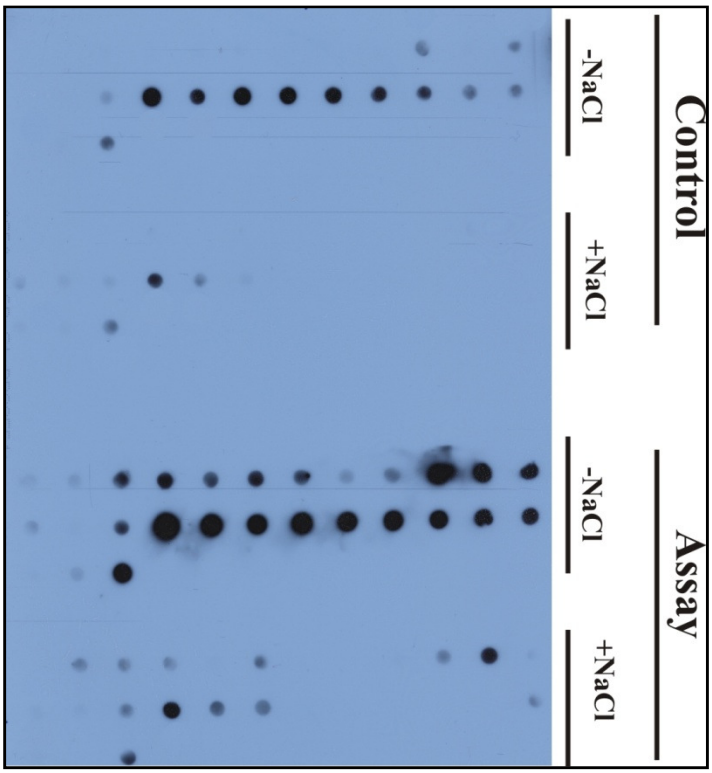


Figure 23: western blot analysis on on-membrane ubiquitination reaction. The ubiquitination reaction was performed on total extract protein fractionated in first dimension by PF2D liquid-chromatography system.

After 20 minutes of exposition time indicating presence of E3 targets in some fractions. In the control reaction, some spots are also detected and they were considered "background" of the experiment to be subtracted from assay reaction spots to get the positive spots. As these preliminary results are promising, we will further fractionate the putative positive spots in the second dimension by hydrophobic interactions. These new second dimension fractions will be analyzed by ubiquitination assays in order to identified the target protein of the E3 used.

APPENDIX: TDRISR E3 UBIQUITIN LIGASE SHOWS A DROUGHT PROTECTIVE ACTIVITY IN TIGS ASSAY

Through a collaboration with Dr. Patrick Schweizer at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany a functional characterization of *TdRiSR* was performed. Given that the *TdRiSR* transcript was shown to be induced by drought stress (Mastrangelo et al., 2005, De Leonardis et al., 2007), the possible involvement of the TdRiSR protein in the protection of cells from dehydration was investigated by the barley DROUGHT-TIGS (Target Induced Gene Silencing) system (Marzin et al., 2008). In this assay, the effect of the transient induced silencing of a target gene in the leaf exposed to *in vitro* dehydration is assessed by the fluorescence of a stress sensitive protein. Cellular stress or damage in dehydrated leaves is reported by a reduced accumulation of slowly maturing, native red-fluorescing protein DsRed that is known to be sensitive to denaturing conditions. Cells suffering from drought or dehydration stress are potentially damaged in membrane integrity, protein folding, and redox status to name a few of the most prominent problems (Ingram and Bartels, 1996). The biochemical properties of the native red fluorescent protein, DsRed, were exploited to report drought stress severity in bombarded epidermal cells of barley. DsRed is known to require several days for maturation into the fluorescent homo-tetrameric complex in animal and plant systems and to suffer from a shift from its red fluorescence to weak green fluorescence upon denaturation (Baird et al., 2000; Gross et al., 2000). It therefore appeared likely that DsRed fluorescence is sensitive to denaturing conditions imposed by severe drought stress over a period of 4 d. Moreover, enhanced proteolytic activity and cell death will also reduce the amount of mature, fluorescent DsRed.

Figure 24 reports a workflow of the TIGS experiment. Detached leafs are co-bombarded with DsRed-, GFP-expression plus TIGS constructs and 24 hours post bombardment GFP expressing cells are counted to normalize the data. Bombarded leafs are treated with stress and after stress imposition the ratio between DsRed/GFP is calculated in treated leafs and in control leafs to determine the eventual involvement of the target gene in cell protection against dehydration stress.

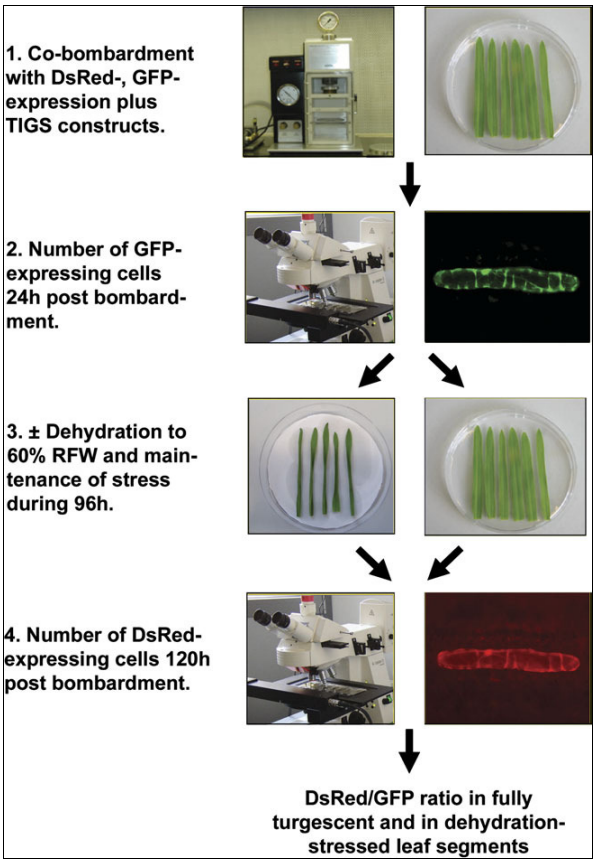


Figure 24: Workflow of DROUGHT-TIGS experiment to investigate involvment of genes in drought stress response in plant. (Image from Marzin et al., 2008).

To perform the experiment a 600bp region of *TdRiSR* coding sequence was cloned in pIPKTA_30 vector in both sense and antisense orientation to induce gene silencing in barley leaves. After bombardment and a dehydration-stress

period of 4 days during which the relative fresh weight of leaves was kept at 60–66% of initial fresh weight, a reproducible reduction of normalized DsRed fluorescence was observed.

In the table below is reported the result of calculation after stress treatment for TdRiSR. A mean value of 100 indicates non involvement of the gene in protecting cells against stress, all values that are significantly below 100 correspond to a role of the gene in protecting cells against stress. As showed in table 3 mean value for TdRiSR gene after 5 experiment is significantly lower than 100 indicating a role of TdRiSR in protecting barley cells during fast dehydration.

Gene	**HarvEST BALSTX Unigene**	**Mean after 5 experiments**	**SD**	**SDM**	**t-test**	**P-value**
TdRiSR	C3H2C3 RING-finger protein like	80.68	9,42698	4,2159	4,5822	0,0101665

Table 3: Results of TIGS-DROUGHT provided by Dr. Patrick Schwaitzer

DISCUSSION

Proteins can be regulated by different mechanisms that, in general, involve posttranslational modifications. Most of these modifications are reversible, such as phosphorylation, acetylation, glycosilation, etc. However the labeling of protein by ubiquitin (Ub) leads to its destruction, making this mechanism irreversible as well as an highly efficient system to control the amount and the activity of key regulatory proteins.

Ubiquitin-dependent degradation plays a key role in controlling several processes that occur during the development and during the response to external stimuli/signals in eukaryotic organisms. Thus, this pathway is involved in hormonal responses (Hellmann and Estelle, 2002), in the response to light (Wang and Deng, 2003), in the control of circadian rhythms (Nelson et al., 2000; Mas et al., 2003) and flowering (Wang et al., 2003), in pathogen resistance (Devoto et al., 2003), cold tolerance (Dong et al., 2006) and salinity response (Hare et al., 2003) to name some. The functional diversity of the Ub/26S pathway is not surprising since the total number of proteins that function in the Ubiquitination pathway in plants is considerably high (approximately 5% of the total protein), being E3 enzymes the most abundant components. This indicates that a large amount of proteins are specifically regulated by ubiquitination. Recently, proteomic approaches allow the purification and identification of a number of proteins that are likely regulated by ubiquitination in plants (Maor et al., 2007; Manzano et al., 2008). However, these analyses does not identify the specific E3 enzymes responsible for recruitment of these ubiquitinated proteins. This knowledge would be critical to define molecular networks and mechanisms that plants exploit to regulate development and interactions with environment. Thus new interesting insights could be obtained by the application of existing methods for studying protein-

protein interactions or by developing new ones specifically devoted to study E3 ligase/substrate–specific interactions (Takahashi et al., 2009; Liu et al., 2009) in order to identify protein targets of interesting E3 ubiquitin ligases as well as regulators of these enzymes.

ROLE OF THE *TdRiSR* GENE CODING FOR AN E3 UBIQUITIN LIGASE ENZYME IN RESPONSE TO ABIOTIC STRESSES

In a previous work the *TdRiSR* gene (formerly *6g2*, Mastrangelo et al., 2005) was identified as upregulated in leaves and roots in response to cold or dehydration (Mastrangelo et al., 2005, De Leonardis et al., 2007). Indeed, it is well known that many proteins are central actors in different signaling pathway, phenomenon known as crosstalk (Chinnusamy et al., 2004). Moreover, the *TdRiSR* transcript was demonstrated to undergo cold induced intron retention of the forth intron.

Beside stress related transcription, the involvement of *TdRiSR* in the plant response to dehydration was demonstrated at phenotype level. Results from application of TIGS-DROUGHT system (Marzin et al., 2008) through the collaboration with Dr. Patrick Schweitzer at IPK-Gatersleben (Appendix) strongly link the silencing of *TdRiSR* gene to a drought-sensitive phenotype indicating a clear involvement of the corresponding protein in drought stress response.

As previously suggested by the presence of a RING-finger motive C3H2C3 type in the aminoacidic sequence, TdRiSR demonstrated a functional activity as E3 ligase in an *in vitro* poliubiquitination assay. Therefore TdRiSR represents the first characterized E3 ubiquitin ligase in wheat. *In vivo* TdRiSR should accomplish this function into the nucleus where a transiently expressed

GFP-TdRiSR was localised in onion epidermal cells. Indeed besides cytosol, the ubiquitin-proteasome system is known to function into the nucleus where plays a major role controlling the initial steps of gene expression, DNA repair and nuclear quality-control mechanisms (von Mikecz, 2006).

In order to identify protein targets of TdRiSR ubiquitin ligase activity or TdRiSR regulators, the yeast two hybrid system has been applied. It allows to test every possible interactions of the interesting protein with all eventual proteins translated by a suitable expression library (Gietz, 2008). 2HY is often criticized for the high level of false positives among its results (Serebriiskii and Golemis 2008), although often used to study E3 enzymes (Dong et al., 2006; Dhawan et al., 2009, Gray et al., 1999). Indeed identified interactors has to be confirmed by *in vitro* ubiquitination assays to prove the direct activity of E3s on its putative target proteins. Others techniques can also be applied to confirm interactions, like *in vitro* pull down assay and *in vivo* bimolecular fluorescence complementation assay.

In this study TdRiSR was shown to interact in yeast with three proteins: TdJUBEL1, TdVIP2 and a TdMAPK. TdJUBEL1 is the wheat homologous of the barley JUBEL1, a BELL1-related TALE homeodomain protein (Müller et al., 2001). TALE proteins are encoded by the class I and class II *KNOX* genes and by *BELL* genes (Hay and Tsiantis, 2009). Within this family, interactions among proteins of different classes of TALE homeodomain proteins are frequent both in plants and in animals, i.e. JUBEL1 interacts with BKN3 (Muller et al., 2001). *KNOX* genes regulate various aspects of plant development and play a key role in maintaining a pluripotent cell population called the shoot apical meristem (SAM) at the growing tip of seed plants (Hake et al., 2004). Moreover several homeobox genes were also found to be differentially expressed under abiotic stress conditions and to be regulated in a posttranscriptional way (Jain and Khurana, 2008).

TdVIP2 is the wheat homologous of the RING-finger protein VIP2 of *Avena fatua*, this proteins interacts with homologous AfVP1 (Viviparous1) and ABI3 (ABA Insensitive 3; Jones et al., 2000). The *VP1* gene has been cloned from maize and shown to encode a seed-specific transcription factor that activates the expression of a number of maturation-specific genes. Seed dormancy and development are mainly controlled by ABA (McCarty, 1995) thus VIP2 could be involved in seed dormancy regulation and in ABA-mediated pathways in plants.

The TdMAPK identified in this work is very similar to *Arabidopsis* MAPKs belonging to the new family WNK. Until now 10 *Arabidopsis WNK* genes have been identified; although few knowledge are available, WNKs seem to be involved in circadian clock and regulation of flowering time (Nakamichi et al., 2002; Wang et al 2008).

The three TdRiSR interactors accomplish different functional roles into the cells, being a transcription factor, an ubiquitin ligase and a MAPK. Given the peculiar characteristics of these interactors, it could be hypothesize that they are not all target of ubiquitination. When the ubiquitination assay was carried out to verify if the interactors are target of TdRiSR, no ubiquitinated proteins were visualized indicating that TdRiSR does not recognize any target in the reaction mixture. This result is not completely surprising; based on the *in silico* analyses the TdRiSR E3 ligase could require some posttranslational modifications such as phosphorylation or myristoylation to become active or to recognize protein partner. Is known that phosphorylation can also directly regulate the activity of E2s and RING-based E3s (Deshaies and Joazeiro, 2009), an example is the study of APC/C (Lahav-Baratz et al., 200); phosphorylation of budding yeast APC/C subunits by mitotic Cdk enhances ubiquitin ligase activity by a mechanism that appears to involve recruitment of the activator protein Cdc20 (Rudner and Murray, 2000).

To further sustain this possibility, when TdRiSR was expressed in an eukaryotic system (rabbit reticulocite) rather than in *E. coli* a stronger ubiquitination was observed, thus suggesting that the E3 in study could require a posttranslational modification that cannot be accomplished by *E. coli*.

To investigate these hypotheses a modified ubiquitination experiment was carried out to test TdRiSR ubiquitination of JUBEL1 or VIP2 in presence of the MAPK. The result obtained for JUBEL1 showed again that JUBEL1 is not a target of the E3. A very interesting result was obtained when the experiment was performed using VIP2 as target protein. In this case VIP2 was shown to act as an E3 ligase being able to accomplish MAPK-independent polyubiquitination (autoubiquitination as well as ubiquitination of E2 and may be E1). This last result lead us to hypothesize that VIP2 and TdRiSR could be two functional partner acting together in plant stress response or that the two E3 enzymes could regulate each other to maintain a precise level of active protein into the nucleus. As reviewed by Mazzucotelli et al. (2007) interaction between different actors of posttranslational modifications can occur at any level. Moreover regulation of RING E3 activity can be mediated by ubiquitin conjugation through a separate E3 that differs from autoubiquitination. For example SCF^{Skp2} is downregulated in early G1 phase by APC/C-dependent turnover of Skp2 (Bashir et al., 2004; Wei et al., 2004)

Another hypothesis could be that TdRiSR E3 ligase is active in the first phases of stress response, to propagate stress signal, but later it must be removed from the nucleus by VIP2 to arrest this signal. Accordingly with this hypothesis the expression profile of TdRiSR transcript is present at a basal level even in non stressed conditions (Mastrangelo et al., 2005).

Although the variety of cellular functions of the three TdRiSR proteins found to interact with stress related–TdRiSR, they are all components of a molecular network affecting plant architecture and development, even if at

different stages of the plant cycle. Instead, no one is known to be involved in plant response to abiotic stress. Even if this can not be *a priori* excluded, the overall results highlight that TdRiSR could mediate a relationship between tolerance to abiotic stress and regulation of plant development.

Links between plant growth, plant architecture and environmental conditions have been amply reported (Cattivelli et al., 2008). It is well known that through hormonal signaling plants synchronize their life cycles with environmental changes by perceiving temperature and photoperiod and upon vernalization (Patel and Franklin, 2009; Myster and Moe, 1995; Henderson and Dean, 2004). In case of environmental constraints, growth reduction is the first response of plants to abiotic stresses both as tolerance mechanism and as symptom of damage. Plants can alter their developmental plans or morphology of their organs to ensure survival and reproduction. In *Arabidopsis,* for example, flowering time is accelerated by elevations in ambient temperature through induction of the floral integrator FLOWERING TIME (FT; Balasubramanian et al., 2006; Lee et al., 2008), while low temperatures result in a dwarfed and compact rosette habit, with leaves displaying increased thickness (Atkin et al., 2006).

Crop plants are sensibly affected by environment constrains. For example, comparison of cereal plants cultivated in irrigated conditions or non irrigated often underlie differences in growth and development about heading date (earlier in non irrigated condition), plant height (higher in irrigated condition) and biomass, these differences strongly impacting on yield, grain weight and number of kernels per m^2 (Fischer and Wood, 1979; Rizza et al., 2004). Reproductive phases are the most sensitive, thus in cereal abiotic conditions affect the setting of grain number (Slafer and Araus, 1998).

Taken together the findings of this work can suggest that the role for TdRiSR E3 ligase in durum wheat stress response might go through regulating or remodeling plant development and architecture in response to stress.

A NEW HIGH-THROUGHPUT SYSTEM TO IDENTIFY E3 LIGASE TARGET

Identifying novel target proteins regulated by ubiquitination is important in understanding the biology of these processes and this will be one of the next challenges in furthering the study of the ubiquitin pathway. Although several proteomic studies have been carried out in yeast, humans and plants to identify ubiquitinated protein (Peng et al., 2003; Vasilescu et al, 2007; Maor et al, Manzano et al., 2008) they were carried out without regards to specificity of the E3 enzymes in recruiting target protein. More recently systems for studying ubiquitination with regards to E3 specificity were developed evaluating few E3 enzymes (Takahashi et al., 2009; Liu et al., 2009). Thus, new high throughput systems to achieve this goal are needed.

In this work, during an EMBO fellowship training in the Dr. del Pozo´s lab, a proteomic approach to identify E3 target proteins on whole proteome was developed in *Arabidopsis*. An ubiquitination reaction by a salt induced E3, performed on a PVDF membrane where the PF2D-fractionation of the whole proteome was spotted, identified protein fractions containing potential E3 target. Even if these are preliminary results - a further fractionation and ubiquitination assays and MS identification of positive proteins are necessary – the effectiveness of transfer of fractionated proteins to PVDF membranes for immuno-analyses (Lopez-Torrejon and del Pozo, submitted) and partially re-naturalization of proteins for biochemical analyses (Lopez-Torrejon and del Pozo, unpublished) have been demonstrated. Therefore this approach could provide a powerful system to study E3 mediated ubiquitination.

PERSPECTIVES

Both the expression profiling (Mastrangelo et al., 2005, De Leonardis et al., 2007) and the results of the TIGS-drought system (Appendix) indicate the involvement of TdRiSR in the plant response to abiotic stresses, in particular to dehydration tolerance. On the other hand, this work has led to the identification of three putative interactors of the TdRiSR E3 ligase, all proteins involved in processes affecting plants development.

Further studies will be required to better understand the relationship between the role of TdRiSR in cellular protection against dehydration and its interactions with regulators of plant growth and development. Therefore, in the next future: the interactions will be verified *in vivo* by bimolecular fluorescence complementation assay; gene expression of the interactors and of the *TdRiSR* gene in response to cold/drought stress or to the signal molecule ABA will be analysed to understand correlation between protein activity and transcript abundance.

Transgenic barley plants overexpressing the wild type TdRiSR E3, or a form mutated in the myristoylation signal region or in the phosphorylation targeted peptide as well as a RNAi line will be generated to better understand the role of this E3 and its eventual posttranslational modifications.

Finally in a broader view, provided that these transgenic materials will show improved performance in stressed environments, new generations of transgenic plants could be developed exploiting these insights as well as the increased knowledge on mechanisms affecting post-transcriptional and post-translational events. On these bases, pyramiding of key regulators of post-

transcritpional/translational events affecting plant stress response could lead us to obtain stress-resistant crop species.

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