The plant orthologue of p62/SQSTM1 links response to nutrients deprivation with the process of selective autophagy

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Abbreviations: AD, activation domain (of GAL4); AO, acridine orange; BD, DNA binding domain (of GAL4); CFP, cyan fluorescent protein; dps; days post sowing; EGFP, enhanced green fluorescent protein; LC3, light chain 3; LIR, LC3 interacting region; LSU, low sulfur upregulated; NES, nuclear export signal; NLS, nuclear localization signal; PB1 domain, Pho and Bem1 domain; UBA domain, ubiquitin binding domain; UBL, ubiquitin-like; Y2H, yeast two-hybrid; YFP, yellow fluorescent protein; ZZ domain, ZZ-type zinc finger domain;

Running title: Joka2 is a selective autophagy receptor in plants

Abstract

Two main mechanisms of protein turnover exist in eukaryotic cells: ubiquitin-proteasome system and autophagy-lysosomal pathway. Autophagy is an emerging important constituent of many physiological and pathological processes, such as response to nutrient deficiency, programmed cell death and innate immune response. In mammalian cells the selectivity of autophagy is ensured by the presence of cargo receptors, such as p62/SQSTM1 and NBR1, responsible for sequestration of the ubiquitinated proteins. In plants there have been no selective cargo receptors identified yet. The present report indicates that a structural and functional homologue of p62/SQSTM1 does exist in plants. The tobacco protein, named Joka2, was identified in yeast two hybrid search as a binding partner of a small coiled-coil protein, a member of UP9/LSU family of unknown function, encoded by the *UP9C* gene strongly and specifically induced during sulfur deficiency. The typical domains of p62 are conserved in Joka2. Ability to homopolymerize and to interact with a member of the ATG8 family argue for the Joka2 role in plant selective autophagy. Moreover, presence of Joka2-YFP in both, cytosolic speckles and the nucleus is in agreement with the recent evidence for nuclear-cytosolic shuttling of p62 in mammalian cells. The *Joka2* expression was up-regulated in roots but not in shoots of tobacco plants grown for two days in nutrient deficient conditions. The above results and the observation that the tobacco seedlings overproducing Joka2-YFP were more tolerant to nutrient deficiency than the seedlings of the parental line suggest that the selective autophagy is an important part of plant response to such environmental stress.

Introduction

Autophagy, or "self-eating" is an ubiquitous catabolic process in eukaryotic cells. Although it was first described about 40 years ago, our molecular understanding of this process started only about a decade ago [1, 2]. The best characterized type of autophagy, macroautophagy, occurs in a wide range of eukaryotes including mammals, plants and fungi, and leads to the degradation of portions of the cytoplasm, which may include cell organelles. During this process a double membrane structure, called autophagosome, sequesters the cargo (e.g. cell material such as organelles, soluble cytosolic proteins and protein aggregates) for degradation. Subsequently, the outer membrane of the autophagosome fuses with the vacuole membrane resulting successively in uptake of the cargo enclosed by the inner autophagosomal membrane (the autophagic body) in the vacuole, the degradation of the cargo and the release of the products for reuse. The membrane origins of autophagosomes are unclear and may involve multiple sources, including the endoplasmic reticulum, Golgi apparatus, mitochondria and plasma membrane [3-6]. At least 34 various proteins, which transiently associate and act in a hierarchical order during autophagosome assembly, have been identified so far. Genes encoding most of these proteins (autophagy related genes, *ATG*) have been found in screenings of yeast mutants defective in autophagy [7]. The process and core molecular machinery components are evolutionarily conserved [8], however the higher eukaryote autophagy pathway might require more elaborate molecular machinery, including factors that are absent in yeast. The human autophagy system has a tremendous influence on protein homeostasis and involves multiple protein-protein interactions [9]. It is needed for appropriate response to nutrient stress, innate and adaptive immunity and autophagic cell death. Malfunction of autophagy has been linked to a wide range of human pathologies, including cancer, different neurodegenerative diseases, immunological disorders and pathogen infection [10-12]. Autophagy is also important during development of mammals, flies and warms [13].

The autophagy specific ubiquitin-like (UBL) proteins of the ATG8 family (known also in mammals as LC3 or GABARAP) are central regulators of autophagosome assembly, maturation and lysosomal fusion. In addition, interaction of the conserved surface of ATG8 with a conserved hydrophobic W/YXXL/I motif (referred as LIR region) in cargo receptors is necessary for the selective cargo recruitment to the autophagosomes [14, 15]. In mammals, at least two proteins, p62/SQSTM1/Sequestosome-1 and NBR1, can function as cargo receptors (or cargo binding proteins) in autophagic clearance of protein aggregates [16-19]. The published data are mostly available for p62, which itself is degraded by autophagy [20, 21]. The p62 is found in cellular inclusion bodies together with polyubiquitinated proteins, in protein aggregates that accumulate in various chronic, toxic, and degenerative diseases [21-24]. The p62 protein mainly facilitates the autophagic clearance of the

aggregates of the ubiquitinated proteins, however, it is also capable of binding nonubiquitinated proteins such as, TRAF6 [25, 26], ALFY [27] or Keap1 [21].

Recent identification of novel selective cargo receptors named SEPA-1 [28] and EPG-2 [29] in *Caenorhabditis elegans*, which are located to the autophagosomes but apparently lack evolutionary conserved structural homologues in mammals, shed more light on the autophagy process in metazoans. One can imagine that other groups of multicellular organisms might contain multiple adaptor proteins mediating a rapid selective autophagic degradation of the unwanted, presumably aggregated, but not ubiquitinated proteins.

Autophagy is a well-known process in yeasts and animals but it has only been recently established in plants. Studies of autophagy in plants are greatly facilitated by the functional and structural conservation of ATG proteins [30,31, 32]. Similarly to the yeast and metazoan systems the plant ATG8 proteins are critical components of the autophagy pathway, therefore in many studies the ATG8-GFP fusions have been used as markers of autophagosomes in plants [33-38]. The lack of obvious phenotypes of *atg* mutants grown under nutrient-sufficient conditions suggested that the autophagy was not essential for plants. However, more detailed studies revealed that the *atg* mutants senescent earlier and were hypersensitive to nitrogen starvation and carbon limitation [35, 36, 39-41]. Moreover, it was demonstrated that the autophagy could be induced by treatment of plants with hydrogen peroxide or methyl viologen [42]. Recently, it was established that under nutrient-deficiency RUBISCO and whole chloroplasts were delivered to the vacuole by autophagy and degraded [43, 44]. In general, autophagy in plants seems to be involved in nutrients recycling. It provides substrates during nutrients deprivation and acts as a cell survival mechanism through recycling cell waste. On the other hand, a number of other evidence indicate that a constitutive basal autophagy occurs also under normal growth conditions [45, 46]. Moreover, it was recently found that autophagy operates a negative feedback loop modulating NPR1-dependent salicylic acid signaling and that this negative feedback is necessary to limit excessive senescence and the programmed cell death in response to pathogen infection [47].

It was commonly believed that in plants no selective autophagy receptors exist and only the core molecular autophagy machinery operates [32, 48]. However, we demonstrate that the Joka2 protein from *Nicotiana tabacum* actually is a structural and functional homologue of p62/SQSTM1. The *Joka2* gene was induced in tobacco roots but not in the shoots during nitrogen (N) or sulfur (S) deficiency. The overproduction of Joka2 improved plant performance in both, normal growth conditions and during nutrients shortage. We propose that Joka2, by analogy to p62 - the selective cargo receptor from the mammalian system, participates in the process of selective autophagy in plants. Our data indicate a link between S-deficiency response and the process of autophagy, and associate for the first time the selective autophagy cargo receptor with plant response to nutrients deprivation.

Results

Identification of tobacco Joka2 as a partner of UP9C

The UP9C protein belongs to the family of UP9/LSU-like proteins present in many plant species [49-51]. Analysis of transgenic tobacco plants with silenced expression of *UP9*-like genes (due to expression of *UP9C* in the antisense orientation) strongly argues for the significant role of UP9/LSU in regulation of plant response to S-deficit [50]. This function is possibly mediated by protein-protein interactions. We previously identified 17 clones encoding putative partners of UP9C originated from the cDNA library prepared from *Nicotiana tabacum* plants grown for 2 days in S-deficient conditions (GenBank Accession No: GU066878–GU066894). Surprisingly, the similar yeast two-hybrid (Y2H) experiment with the cDNA library prepared from *N. plumbaginifolia* seedlings grown in normal (nutrient sufficient) conditions resulted in identification of only three clones denoted pJoka2, pJoka8 and pJoka20 (**Fig. 1A**) encoding different proteins than those identified from *N. tabacum* library. Database searches and location of characteristic domains within the predicted open reading frames of Joka2, Joka8 and Joka20 allowed either for identification of a corresponding protein previously known in tobacco (the case of Joka20) or for identification of homologues in other plant species (the case of Joka2 and Joka8). The Joka20 protein was identified as L7/L12, a nuclear encoded component of chloroplast ribosomes [52]. The Joka8 protein appeared to contain a basic helix-loop-helix (bHLH) motif and was classified as a member of a huge family of bHLH transcription factors [53]. Initially, no function to Joka2 could be assigned but it appeared to contain two well-characterized domains: Phox/Bem 1p (PB1; PFAM:00564) and ZZ-type zinc finger (ZZ; PFAM:00569). The *Joka2* open reading frame was incomplete since no translation initiation and no translation stop codons were present in the cloned cDNA fragment.

Interactions between UP9C and the identified partners were confirmed by the "pull-down" assay. In order to check which of the two well-characterized protein domains, PB1 and ZZ, present in Joka2 is involved in the interactions, the DNA fragments containing each of the domains were cloned separately into expression vectors. From the results shown in **Fig. 1B** and **Table 1** it can be concluded that the ZZ domain is most probably responsible for interaction with UP9C. Additionally, we demonstrated that the LSU1 protein encoded by At3g49580 gene of *Arabidopsis thaliana* is able to interact with Joka2 (**Table 1**). This finding indicates that despite the relatively limited sequence similarity and the relatively weak interaction between UP9C and LSU1 (**Table 1**), the protein features responsible for interactions with Joka2 are conserved in the UP9/LSU family.

It was reasonable to anticipate that the Joka2 protein is longer than the one deduced from the cloned sequence, therefore we focused on cloning of the full length cDNA from *N. tabacum*. Using the RT-PCR, 5'-RACE and 3'-RACE methods the cDNA encoding the full-length protein have been cloned. In addition to PB1 and ZZ domains, the deduced protein contained also a duplication of the

ubiquitin associated domain (UBA/TS-N; pfam00627). All subsequent experiments were carried out only using plasmids containing the *N. tabacum* cDNA.

Joka2 is a homologue of p62/SQSTM1 and forms homomers

Proteins with a similar to Joka2 layout of domains exist in other eukaryotic organisms, both animals and plants (**Fig. 2A**). The best characterized protein with a comparable arrangement of domains is the human protein p62/SQSTM1 called also Sequestosome-1, A170 or ZIP, which is a multifunctional protein implicated in several signal transduction pathways and is required for autophagic clearance of protein aggregates. It acts as a selective autophagy receptor by interacting with both, ubiquitin coniugated to the target proteins and the ATG8 proteins present on the autophagosome [14, 19, 54]. As shown in **Fig. 2B**, p62 comprises a N-terminal region that includes PB1 domain (residues 20-102) and zinc finger (ZZ, residues 122-167), a central region containing LIR (LC3-interacting; residues 337-343) and KIR (Keap 1-interacting; residues 346-359), and C-terminal region encompassing a ubiquitin-associated domain (UBA, residues 391-436) [21]. A nuclear export signal (NES) and two basic monopartite nuclear localization signals (NLS1 and NLS2) are located between residues 303-320, 186-189 and 264- 277, respectively [55]. The three domains (PB1, ZZ, UBA) characterized in p62 can be also found in its plant homologues. However, the plant proteins are generally of longer size, have several candidates for LIR motifs, NLS regions and larger (duplicated) UBA domain (**Fig. 2B**; **Suppl. Table 1**).

The formation of Joka2-Joka2 dimers has been demonstrated in two independent Y2H experiments for either NpJoka2 or NtJoka2 (**Fig. 3B**). The region necessary for such interaction appeared to contain PB1 domain and was mapped to the first 228 residues of NpJoka2 (see: pJK6, **Suppl. Table 2**) and the first 422 residues (see: pDEST22/PB1 and pDEST32/PB1, **Suppl. Table 2**) of NtJoka2. These results are consistent with the data for p62 showing that PB1 domain is crucial for the formation of multimers.

Joka2 interacts with ATG8

Despite identification of many authophagy-associated (ATG) genes in plants no reports on the presence or a potential function of any of the p62/Joka2 homologues could be found in the literature. Therefore, it was important to confirm that Joka2 indeed posses the most crucial and well-established features of p62, binding to ATG8/UBL proteins. In higher eukaryotes, both mammals and plants, there is a large family of ATG8/UBL-like proteins. For example, in human genome there are six genes encoding ATG8 family members [9], while in *A. thaliana* nine genes encoding ATG8 family (AtATG8a - AtATG8i) are present and expressed [38, 56]. Function of AtATG8f under both favorable growth conditions and under starvation stresses was previously investigated [33, 34] thus this isoform was a candidate of our choice for testing if the members of this family would interact with Joka2. Two

(TC107227 and TC99613) out of 15 known tobacco EST sequences encoding full length proteins corresponding to ATG8 (**Suppl. Table 1**) might encode a counterpart of AtATG8f. Both of them encoded almost identical proteins with only two amino acids extension at the C-terminus in the case of TC107227. This cDNA, referred below as NtATG8f, has been cloned and used in Y2H experiment to investigate its potential interaction with Joka2. In fact, the results shown in **Fig. 3** clearly indicated that such interaction takes place *in vivo* when the full length NtATG8f and NtJoka2 proteins were used. It was also possible to limit the region of NtJoka2 necessary for the interaction with NtATG8f to the residues 1-751 (out of 843 in total). However, in our hands interaction of the trimmed down NtJoka2 with NtATG8f could only be observed if the former was present in the BD-plasmid but not in the AD-plasmid (**Fig. 3A**). The LIR motif of mammalian p62, responsible for binding to ATG8/UBL proteins, is located between ZZ and UBA domains. In plant Joka2/p62 proteins several potential LIR motifs might be predicted (**Fig. 2B**). Our results do not yet allow to distinguish, which of these LIR motifs indeed function as binding sites for ATG8/UBL proteins.

Joka2 localizes in cytosolic speckles and in the nucleus

Tobacco J4 and J5 lines containing *Joka2-YFP* and *Joka2-CFP* expression cassettes, respectively, were obtained by Agrobacterium-mediated transformation of LA Burley 21 with the binary pJ4 and pJ5 plasmids (**Suppl. Table 2**). Expression of the transgenes in the selected lines was confirmed (not shown) and the J4-1, J4-2, J4-10, J5-1, J5-2, J5-3 and J5-6 lines were selected for further experiments, including monitoring of Joka2 localization *in planta*. For these purposes the seeds were germinated and seedlings were maintained in S-sufficient (nS) or S-deficient (S-) liquid media, and in H_2O. Observations were performed on the 10th, 17th and 33rd day-post-sowing (dps). Localization of Joka2-YFP in J4-1 seedlings is shown as a typical example (**Fig. 4**), since no difference between Joka2-YFP and Joka2-CFP were observed. Distribution of Joka2-YFP in the roots was different in different media (**Fig. 4A**). In the root elongation zone of the nutrients deprived seedlings (maintained in H_2O) Joka2-YFP was present in multiple punctated structures, contrary to a single larger spot of the fusion protein observed in seedlings grown in nS medium. Interestingly, in the roots of older seedlings (33 dps), regardless from the medium, the Joka2-YFP signal co-localized with DAPI-stained nuclei. In contrast to the roots, distribution of Joka2-YFP in leaflets, hypocotyls and the root tips was not affected by nutrients availability (**Fig. 4B**). In these plant parts, Joka2-YFP formed a few cytosolic spots, frequently located near the nucleus. Interestingly, a clear boarder between the shoot and root parts was evident when monitoring distribution of the fusion protein.

The striking punctated location of Joka2-YFP in the roots of seedlings grown in H_2O was further investigated using Acridine Orange (AO) staining (**Fig. 4C**). The AO dye has been previously used to stain the autophagosomes in mammals [57], giving a red signal for all acidic compartments but it gives also a green signal with DNA. During this experiment, in addition to J4-1, two tobacco lines

served as controls: (i) LA Burley 21, which was a parental line for all transgenic lines used in this study and (ii) the transgenic AB5 line producing EGFP. As expected, staining of LA Burley 21 and AB5 seedlings with AO displayed green fluorescence with cytoplasmic and nuclear components but, in contrast to J4-1, the seedlings of LA Burley 21 and AB5 did not display considerable red fluorescence, suggesting formation of acidic autophagolysosomal structures mostly in the seedlings overexpresing Joka2-YFP. The signal of Joka2-YFP co-localized with the red signal of AO. Such co-localization was apparent only in the J4-1 line, what indicates that the large speckles of Joka2-YFP localize indeed within acidic compartments, what in turn, is a characteristic feature of autophagosomes/lysosomes. Summarizing the localization studies, Joka2-YFP was observed predominantly in the cytoplasmic acidic speckles but also, in later stages of seedlings development, in the nucleus. Application of protease inhibitor, E64d, resulted in increased number of Joka2-YFP foci (**Fig. 4D**) due to inhibition of the autophagic degradation of the protein.

Sulfur and nitrogen deficiency affects expression of *Joka2* and *ATG8*

Expression of *NtJoka2* and *NtATG8f* was monitored in different parts (young leaves, mature leaves, stalks and roots) of two-month-old LA Burley 21 plants grown for 2 days without sulfur (S-) or without nitrogen (N-) or in the control (nutrient-sufficient; nS) conditions. The semiquantitative RT-PCR (sqRT-PCR) indicated that *ATG8f* and *Joka2* expression was up-regulated in roots but not in any shoot parts after two days of growth in either S- or N- conditions (**Fig. 5**). The expression of *UP9C* was used as a control and, as previously reported [50], this gene was induced in all parts of the plants in S- but not in N- conditions. Interestingly, what was not reported before, a slight reduction of *UP9C* expression was observed in N- in mature leaves as compared to nS. It is a striking observation, however the significance of such regulation is unclear to date.

Overexpression of Joka2-YFP or Joka2-CFP in tobacco improves seedlings development

Transgenic lines of tobacco abundantly and constitutively overproduced the fusion proteins and this might result in the excess of the Joka2 product in comparison to the parental line. To check how this overproduction effects the plant performance we monitored seedlings growth using several transgenic lines. It appeared that seedlings from most of the checked J4 (2 out of 3 tested) and J5 (2 out of 4 tested) lines grew better than the control seedlings in the regular medium. The seedlings of the parental line, LA Burley 21 and the seedlings of the previously mentioned AB5 line (overexpressing EGFP) were used as controls (results not shown). To investigate further this phenomenon two transgenic lines, J4-1 and J5-3, were germinated in parallel with the above mentioned controls in water. The phenotype and chlorophyll contents were monitored at 14 dps. The results indicated that J4-1 and J5-3 lines were much less sensitive to nutrients deprivation and had higher chlorophyll contents than the control seedlings (**Fig. 6**). To exclude the possibility that the performance of the

seedlings is linked to the seeds mass, the weight of 200 seeds from each line was checked in triplicates. The following average values were calculated: 14.87 ± 0.23 mg for LA Burley 21, 14.13 ± 0.66 mg for AB5, 14.17 ± 1.83 mg for J4-1 and 12.53 ± 0.83 mg for J5-3. This result indicated that J4-1 and J5-3 lines did not produced larger or heavier seeds that the control lines. Therefore, difference in seedlings performance must depend on other factors, for example on efficiency of usage of the limiting nutrients.

Discussion

Autophagy is a highly selective process implicating cargo receptors in removing protein aggregates and damaged or excess organelles. A number of ubiquitin–binding proteins containing also a short LIR motif exist in mammals. Among them the best characterized is p62/SQSTM1. It links autophagy and proteasome systems by competing for common substrates (ubiquitinated proteins) with other ubiquitin binding proteins [48, 55]. The structural motifs of p62 are shared by its plant homologues what suggests that their function is also conserved. In this study we focused on the plant protein Joka2 and demonstrated that it is an orthologue of the mammalian autophagy cargo receptor, p62/SQSTM1. Based on domains conservation and on such experimental evidence as, formation of Joka2-Joka2 homodimers, interaction with NtATG8f, subcellular localization pattern and increased number of Joka2-YFP speckles in response to E64d treatment we propose that Joka2 functions as a selective autophagy cargo receptor in plant cells. Thus, the selective authophagy functions in plants in a similar way as in mammalian cells and involves both, the core molecular machinery as well as the selective cargo receptor(s).

Only one isoform of Joka2/p62-like protein was identified in *N. tabacum*. In addition, we were able to identify only one copy of *Joka2/p62*-like gene in *A. thaliana*, At4g24690. Additional Blast search pointed out the product of the same *A. thaliana* gene as a homologue of the mammalian NBR1 (not shown). This result suggests that Joka2 might be the only autophagy cargo receptor for protein aggregates in plants, in contrast to the situation in mammals, where at least two of them (p62 and NBR1) are present [17]. Intriguingly, none of the several available T-DNA insertional mutants (SALK_053992, SALK_135513, SALK_144852, GK536D03.01) within At4g24690 gene appeared to be homozygous. Despite our intensive efforts we failed in obtaining homozygous T-DNA insertional mutants in the next generations and observed strongly reduced seed germination efficiency in these lines (Zientara-Rytter, Sirko – unpublished). Although it is tempting to speculate that At4g24690 might encode an essential protein, needed at the stage of seed germination, the experimental evidence supporting this notion is still limited.

In our experiments (**Fig. 4)** Joka2-YFP was present in both, the cytosolic acidic speckles (co-stained with AO) and in the nucleus (co-stained with DAPI). Mammalian p62 can also shuttle

continuously between cytoplasm and the nucleus and it has nuclear localization (NLS) and nuclear export signals (NES). Shuttling of p62 between both compartments is modulated by phosphorylation and aggregation of p62 [27, 55]. Unlike the cytosol, where two complementary proteolytic systems, lysosomes and proteasomes, are responsible for protein degradation, the nucleus is believed to have only the proteasomal system. Therefore, it was suggested that in the nucleus p62 could facilitate the recruitment of protein aggregates to the proteasome [55]. Other researchers suggested that p62 can serve as an adaptor for proteasomal degradation of certain ubiquitinated proteins and that it has an ubiquitin-independent role in degradation of some autophagy substrates [58, 59]. On the other hand, it has been postulated that p62 can be implicated in two different, but not mutually exclusive, mechanisms of the cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. At time when autophagy operates at normal rate, p62 serves to deliver the ubiquitinated proteins for autophagosomal destruction. However, in the situation when autophagy is impaired, p62 prevents the proteasomal degradation of some proteins by competing with other ubiquitin binding proteins, which facilitate proteasomal degradation [60, 61]. In any case, the ability reside in both the autophagosomes and the nucleus suggests that p62 serves a complex role. Recently, a reporter system to monitor autophagy in mammalian cells based on p62 has been proposed [62]. According to the authors the GFP-p62 performed the best among the three tested reporter fusions (GFP-LC3, GFP-NBR1 and GFP-p62) and was the most useful in screening for compounds or conditions that affected the rate of autophagy. The transgenic tobacco plants described in this work, which overproduce Joka2-YFP and Joka2-CFP fusion proteins might be also a useful tool for studying the process of autophagy in plants.

Some our results, including organ-specific regulation of Nt*Joka2* and Nt*ATG8f* transcription by N- and S-deficiency, interaction between NtJoka2 and UP9C, and increased tolerance of J4 and J5 plants to nutrients deficiency strongly suggest that Joka2 is involved in plant response to nutrients deficit. As shown in **Fig.5** transcription of *Joka2* and *ATG8f* is apparently induced in tobacco plants grown in either N- or S-deficiency. Interestingly, in the conditions of our experiments (two days of nutrients deficit) expression of both genes was up-regulated only in the roots but not in any shoot parts. This observation might be important in identification of the regulatory elements of the long-distance signaling of nutrients availability and of the overall plant nutritional status. The autophagy process in plants occurs under several conditions, such as a general response to different abiotic stresses, a general mechanism for regulation of programmed cell death in response to pathogens and during developmentally regulated programmed cell death. This is essentially in concordance with much better characterized regulation of this process in mammals, where autophagy is controlled by pathways that interpret the status of cellular energy (AMP-dependent protein kinase, AMPK), nutrients (target of rapamycin, TOR) and growth factors such as insulin [9]. Although more studies are needed to understand the regulation of this process some previously considered candidates for regulators of *ATG* genes in plants include hormones such as cytokinin [34] and ABA [63].

The better performance of the transgenic tobacco lines containing increased amounts of Joka2-YFP and Joka2-CFP in comparison to the control plants might be an indication of better recycling of the waste due to the enhanced autophagy in these lines. The similar growth effects were observed in *A. thaliana* overproducing ATG8f-GFP-HAtag fusion [34].

The Joka2 protein was identified as a partner of UP9C protein. The function of UP9C is unknown but it has been recently recognized as required for adequate plant response to sulfur deficit [50]. Some UP9/LSU-like proteins, especially those encoded by genes strongly induced during sulfur deprivation, might link sulfur deficiency response with selective protein degradation through autophagy or in proteasomes. As demonstrated in our laboratory, UP9C is able to interact with a variety of protein partners, including proteins involved in synthesis and signaling of important phytohormons, such as jasmonic acid and ethylene. Concerning the possible mechanisms, one option would be that UP9/LSU can modulate such properties of Joka2, as subcellular localization, cargo recruitment and/or ability to form multimers or aggregates. The other option is that UP9C could act as an adaptor between the cargo and the cargo receptor (Joka2). This could be a novel regulatory mechanism responsible for the fast functional inactivation or protection of the target proteins. In consequence, the process would ensure the swift plant adjustment to sulfur availability. The above hypotheses are currently under studies in our laboratory.

Materials and Methods

Yeast two-hybrid experiment

The *N. plumbaginifolia* cDNA library in pGAD10 was a kind gift of Dr. Witold Filipowicz, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland. Manipulation of yeast cells and library screening were carried out according to standard protocols (Clontech Yeast Protocol Handbook, PT3024-1). The *Saccharomyces cerevisiae* strain PJ69-4 [64] was used for transformation and approximately 6.5 x 10^6 transformants were plated on selective medium lacking leucine, tryptophan and histidine(SD-LTH). True positive prey clones, after retransformation, were confirmed for their ability to activate the three reporter genes, *HIS3*, *ADE2* and *lacZ*, when cotransforming yeasts with pJK1 as a bait. For simultaneous transformation with the defined "bait" and "prey" plasmids, since just several transformants were sufficient for further applications, the "Quick and Easy TRAFO Protocol" available at [http://www.umanitoba.ca/faculties/ medicine/biochem/gietz/] and the *S. cerevisiae* AH109 strain with four reporter genes (HIS3, ADE2, MEL1 and lacZ) were used.

Immunoblots and "pull-down" assay

Prior to the "pull-down" assay the presence of recombinant proteins fused to GST in the respective bacterial extracts was verified by immunoblots using rabbit polyclonal anti-GST IgG (Santa Cruz Biotechnology Inc., SC-33613) as primary antibody and anti-rabbit IgG conjugated to alkaline phosphatase (Sigma-Aldrich, A3687) as secondary antibody. The "pull-down" assay consisted of the following steps: (i) the protein extract from bacteria producing recombinant His-tagged UP9C was mixed with the extracts from bacteria producing GST-ZZ (the part of Joka2 with ZZ domain), GST-Joka8, GST-Joka20 or GST; (ii) extracts were incubated on ice for 6h with gentle rocking; (iii) proteins were purified in the native condition on Glutathione Sepharose column (GE Healthcare Bio-Sciences AB, 17.0756-01); (iv) Western blots were performed with rabbit polyclonal anti-His IgG as primary antibody and anti-rabbit IgG conjugated to alkaline phosphatase as secondary antibody.

Plant growth conditions and chlorophyll measurment

Seeds of wild plants and transgenic *N. tabacum* (cv. LA Burley 21) were surface-sterilized in microcentrifuge tubes using a vapor-phase seed sterilization method. Shortly, tubes with seeds were placed into a desiccator jar along with two beakers, each containing 50 ml of bleach and 1.5 ml of concentrated HCl. Sterilization by chlorine fumes was continued for 3h. Then, centrifuge tubes containing seeds were placed in a sterile laminar flow hood and left open for 1h. Depending of the experiment, *N. tabacum* seeds were spread into water or on plates with modified 0.5 x Hoagland medium either full or lacking nitrogen (N-) or sulfur (S-). In N- medium the equimolar amounts of KOH, KH_2PO_4 and $CaCl_2$ were used instead of KNO_3, $NH_4H_2PO_4$ and $Ca(NO_3)_2$, while in S- medium the equimolar amounts of $MgCl_2$ replaced $MgSO_4$. The medium was either liquid (in Erlenmayer flasks or hydroponic containers) or solidified with agar (0.8% w/v). The seedlings were incubated in a growth chamber under a long-day regime of 16 h light/8 h dark cycle at 24°C.

For testing the effect of nutrient deficiency on seedlings performance, the sterilized seeds were germinated and seedlings were maintained for 14 days in H_2O. Entire seedlings were collected and the chlorophyll content was measured as previously described [65].

Plant transformation and transient expression

Agrobacterium tumefaciens strain LBA4404 was transformed with each of the binary plasmids listed in **Suppl. Table 2**. The plasmids pJ4, pJ5 were used for stable transformation of *N. tabacum* as previously described [66]. Kanamycin- or hygromycin-resistant plants were selected and self-pollinated to obtain T2 generation.

DNA methods and plasmids construction

Plasmids used in this work are described in **Suppl. Table 2**. Details on their construction are available upon request. Gateway BP recombination by using attB-tailed gene-specific primers or cDNA cloning

into pENTR™/D-TOPO vector and Gateway LR recombination reactions were done as described in the Gateway® Technology – A universal technology to clone DNA sequence for functional analysis and expression in multiple systems (Invitrogen, 12535-019 and 12535-027). Oligonucleotides for PCR, RT-PCR, RACE, and DNA sequencing are listed in **Suppl. Table 3**. All plasmids were checked by restriction digestion and/or by DNA sequencing. Conventional techniques were used for DNA manipulation and *Escherichia coli* transformation [67]. The cDNA synthesis and semiquantitative RT-PCR (sqRT-PCR) were conducted as previously described [68]. RNA isolation and mRNA purification for the 5'- and the 3'-RACE were performed using the SMART™ RACE cDNA Amplification Kit (BD Biosciences-Clontech, 634914) according to the procedure recommended by the manufacturer.

Microscopy methods

Tobacco seedlings grown for 10, 17 or 33 days in water or defined liquid medium were washed and incubated in fluorescent dyes (DAPI or/and Acridine Orange [AO]) for 15 minutes in the darkness at room temperature. After the treatment, seedlings were washed in water (3 times, 5 min each) and observed using a confocal microscope. The cytoplasm and DNA of the AO-stained cells fluoresced bright green, whereas the acidic autophagic compartments and RNA fluoresced bright red. 14-day-old seedlings were incubated in 1μg/ml E-64d protease inhibitor for 1 day. DAPI (Sigma-Aldrich, D9564) was prepared as a 1mg/ml stock solution in DMSO. AO (Invitrogen, A3568) was dissolved in H_2O to 10 mg/ml. E-64d (Alexis, BML-PI107-0001) was dissolved in ethanol as a 10mg/ml stock. The images were obtained at the Laboratory of Confocal and Fluorescence Microscopy at IBB PAS with a Nicon confocal microscope, Eclipse TE2000-E and processed using EZ-C1 3.60 FreeViewer software.

Computer analysis and accession numbers

Similarity searches were performed with BLASTP at NCBI [http://www.ncbi.nlm.nih.gov/BLAST/] or with BLASTN using TGI Database of plant EST at DFCI [http://compbio.dfci.harvard.edu/tgi /plant.html]. Translation of nucleotide sequences was generated at EMBL-EBI [http://www.ebi.ac.uk/services/index.html]. Translation of nucleotide sequences was generated at EMBL-EBI [http://www.ebi.ac.uk/services/index.html]. Multiple sequence alignment was generated by MAFFT ver.5.667 using the E-INS-i strategy [http://timpani.genome.ad.jp/~mafft/server/] [69]. Phylogeny was inferred by programs from the Phylip v3.36 package [70]. Sequences were aligned in Phylip-format using T-coffee [http://www.ch.embnet.org/cgi-bin/tcoffee_parser]. The following programs were accessed through the ExPASy Proteomic Server [http://www.expasy.ch/]: GOR4 [71] used for secondary structure prediction, PSORT [72] used for predicting the localization, SMART [http://smart.embl-heidelberg.de] [73], MOTIFSCAN [74] and EML Server [http://elm.eu.org/] for identification of the protein domains and patterns.

The accession numbers of the sequences used in this study are listed in **Suppl. Table 1**.

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Figure Legends

Figure 1. Interactions of UP9C investigated by Y2H (A) and the "pull-down" (B) assays. (A) The pJK1 and pJK2 plasmids contain *UP9C* in the BD- and AD-vectors, respectively. The yeast strains co-transformed with indicated BD- and AD-plasmids were grown on selective media without leucine and tryptophan (SD-LT), leucine, tryptophan and adenine (SD-LTA), leucine, tryptophane and histidine (SD-LTH) or were screened for β-galactosidase expression (+X-gal). (B) The Western blot shown on the left-hand side verifies expression of the GST-fusion proteins in the extracts from bacteria producing GST-PB1 (2a), GST-ZZ (2b), GST-Joka8 (8) and GST-Joka20 (20); the GST-PB1 protein was not detected. The Western blot shown on the right-hand side shows results of the "pull-down" assay performed as described in Materials and Methods. The results confirm interaction of UP9C with Joka8, Joka20 and the ZZ domain of Joka2; the extract containing His-tagged UP9C protein (C+) was loaded as a positive control and the arrows indicate the positions of the proteins corresponding to the expected sizes of the recombinant proteins: GST-PB1 (53.3 kDa; undetected), GST-ZZ (54.5 kDa), GST-Joka8 (66.5 kDa), GST-Joka20 (42 kDa) and His-UP9C (17.2 kDa).

Figure 2. The family of p62/SQSTM1/Joka2 proteins. (A) The phylogenic tree was constructed using full length protein sequences by the parsimony methods and 100 bootstrap replicates using SEQBOOT, PROTPARS and CONSENS of the Phylip v.3.69 program package. The bootstrap values are given at the respective branches. The accession numbers of the proteins included in the analysis can be found in **Suppl. Table 1**. (B) Characteristic domains present in the p62/SQSTM1/Joka2 proteins from *Nicotiana tabacum*, *Arabidopsis thaliana* and *Homo sapiens*. Proteins and domains are drown in scale. See text for details.

Figure 3. Interactions of Joka2 (A) and scheme of the Joka2 inserts present in the Y2H plasmids (B). AD means fusions of the activating domain of GAL4 with the indicated protein or domain. BD means fusions of the DNA binding domain of GAL4 with the indicated protein or domain. Pluses and minuses indicate the growth and lack of growth, respectively, on the plate shown on the left-hand side. The growth is an indicator of protein-protein interaction. Proteins and domains shown in panel B are drown in scale.

Figure 4. Localization of Joka2-YFP in tobacco seedlings. (A) Joka2-YFP signal in roots of seedlings grown for the indicated numbers of days post sawing (dps) in the indicated conditions: water, S- conditions and full medium (nS). The right-hand pictures are overlays of DNA staining with DAPI and the signal for Joka2-YFP. The seedlings grown in H_2O did not survived till 33 day. (B) Joka2-YFP signal in leaves, hypocotyls and root tips (as indicated by the brackets) in

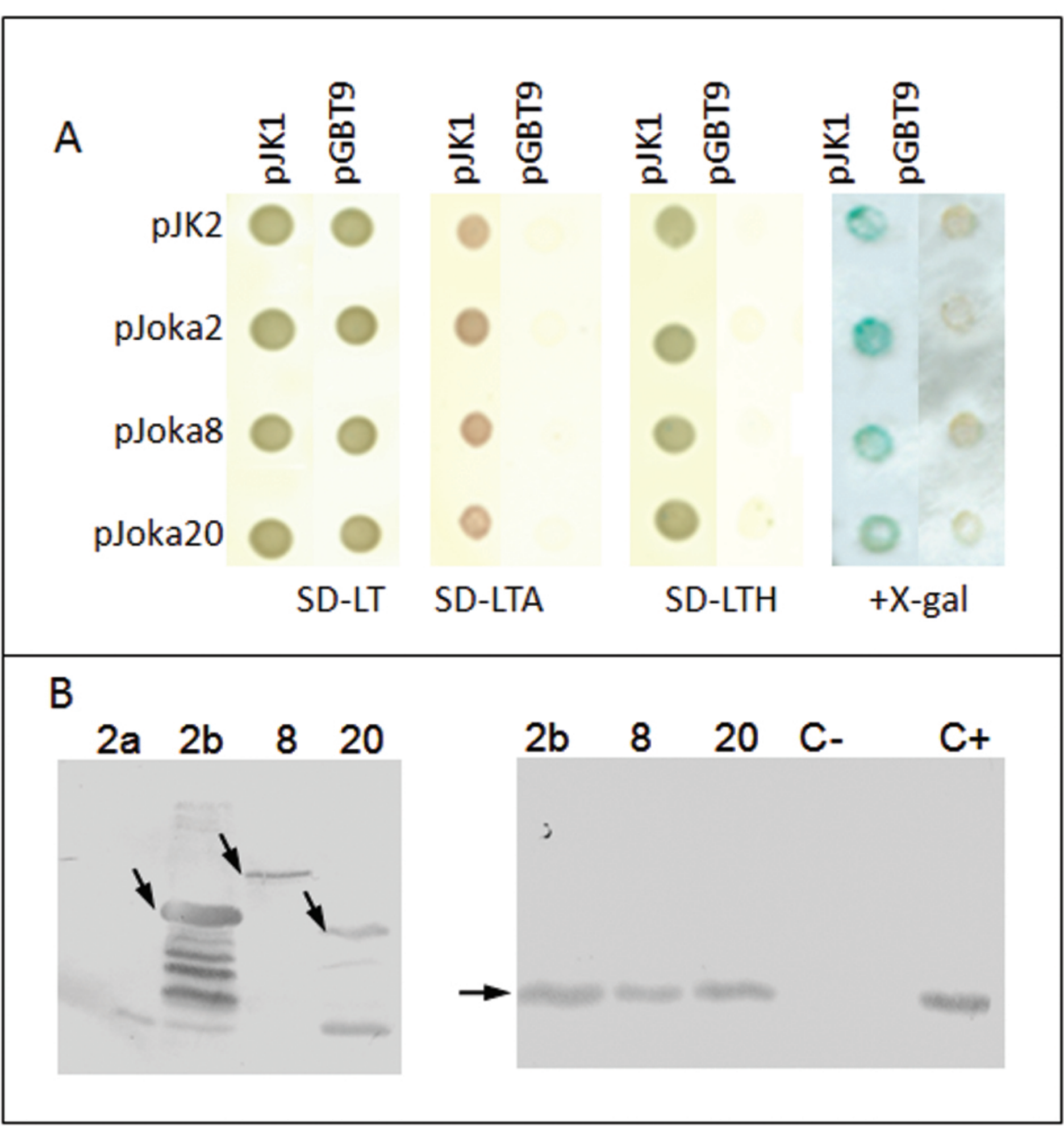
10-day-old seedlings (10 dps) grown in H_2O. Notice, a clear-cut border between the roots (Joka2-YFP abundant) and the stems (Joka2-YFP hardly detected). (C) Co-localization of Joka2-YFP and acidic (AO-stained) compartments. Seedlings of the parental LA Burley 21 (LAB21), transgenic J4-1 (producing Joka2-YFP) and transgenic AB5 (producing EGFP) were grown for 17 days in H_2O. The left-hand panel shows accumulation of Joka2-YFP signal and the green signal of AO (if applied). The middle panel shows the red signal of AO (if present). AO gives the green signal in non acidic environment of the cytoplasm and the nucleus and the red signal in acidic compartments and when bound to RNA. The right-hand panel shows the corresponding tissues without fluorescence. The dashed arrows point the nuclei positions. The acidic speckles (autophagosomes/autolysosomes), pointed by the solid arrows, are present only in J4-1 line and do not overlap with nuclei. (D) Joka2-YFP signal in the root cells in 14-day-old seedlings. Both upper panels show the Joka2-YFP signal, while both lower panels show the corresponding tissues in the transparent view. Both right-hand panels show the control conditions, while both left-hand panels show the tissues after the treatment with protease inhibitor E-64d. The emission wavelengths of the filters are indicated; Trans denotes transparent view.

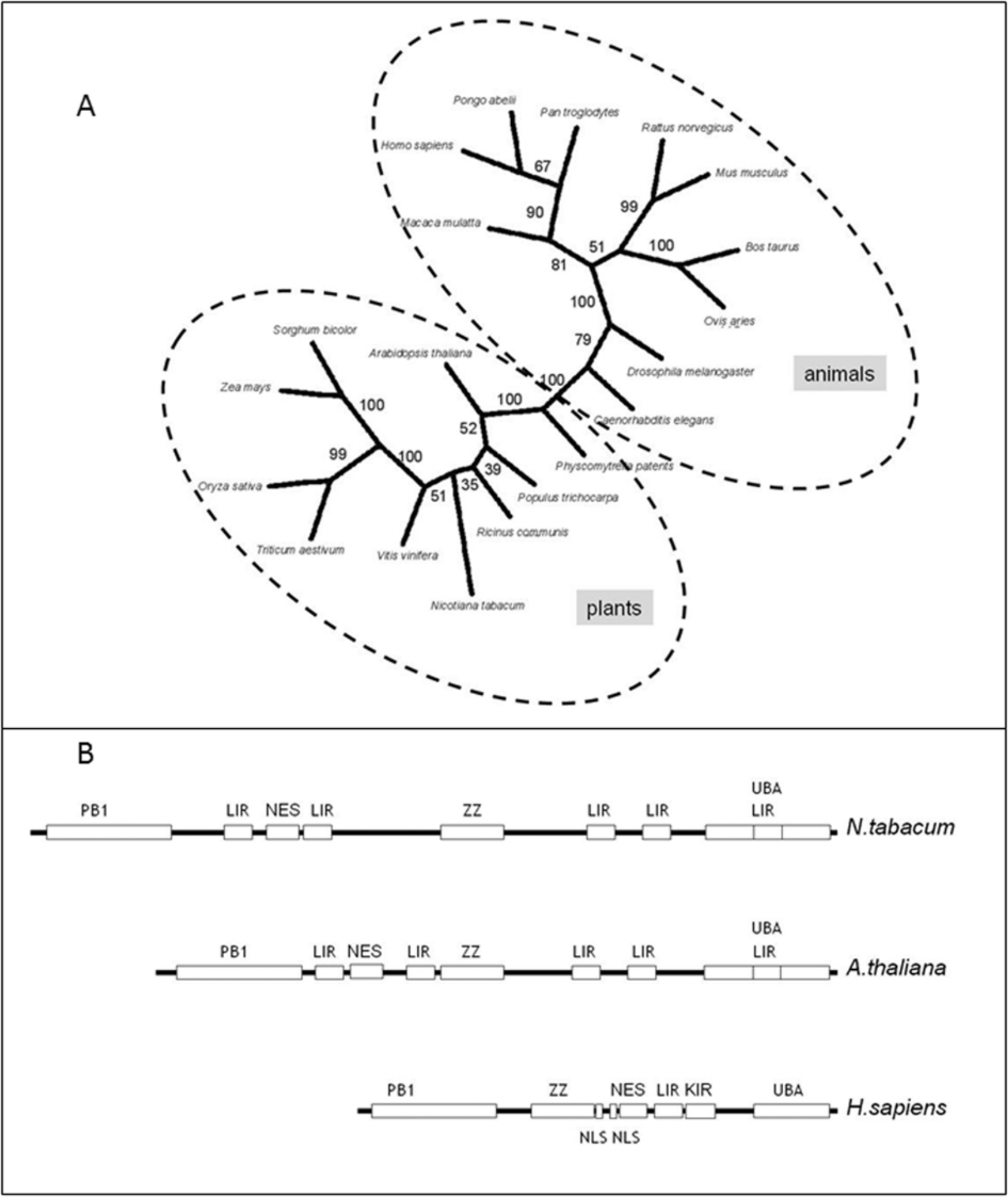
Figure 5. Expression of *Joka2*, *ATG8f*, *UP9C* and *UP9A* in various parts of LA Burley 21 plants. The 8-week-old plants grown in nutrient-sufficient (nS) medium were transferred for two days into S- or N-deficient medium and, as a control, to nS again. Gene expression was monitored by sqRT-PCR. Expression of actin (*Tac9*) served as a control.

Figure 6. Phenotype (A) and chlorophyll contents (B) of tobacco seedlings germinated and maintained for two weeks in nutrient-deficient conditions. The lines overproducing Joka2-YFP (J5-3), Joka2-CFP (J4-1), EGFP (AB5) and the parental line LA Burley 21(LAB21) were used.

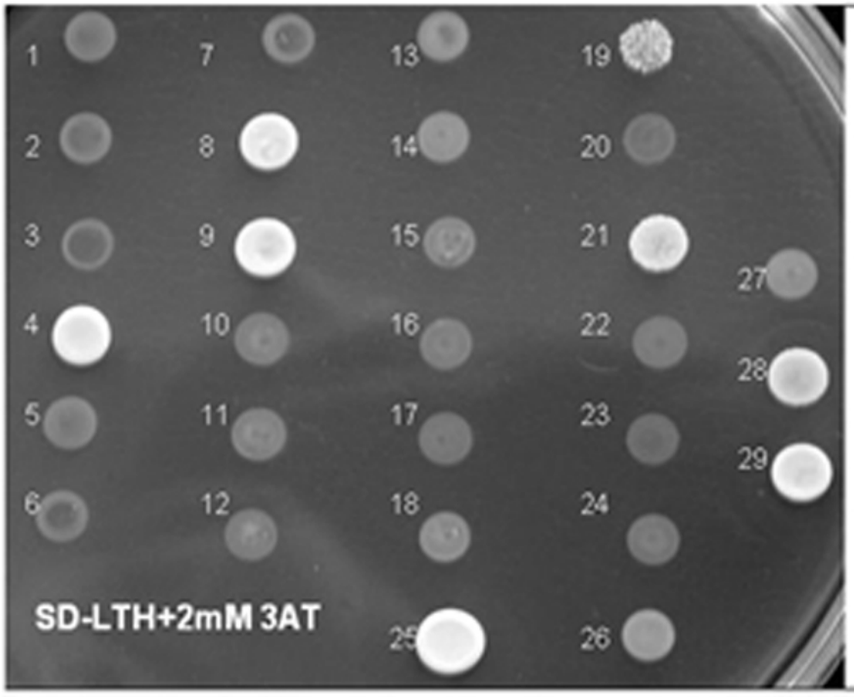
Table 1. Summary of additional interactions examined in the yeast two-hybrid (Y2H) experiments. The interactions were determined by the growth of yeast transformants on the respective selective medium due to expression of the reporter genes *HIS3* and *ADE2*. Descriptions in the brackets indicate the protein encoded by the cDNA present in the respective Y2H plasmids; PB1 denotes the PB1 domain of Joka2, ZZ denotes the ZZ domain of Joka2. The Joka2, Joka8 and Joka 20 are from *N. plumbaginifolia*, UP9C from *N. tabacum*, and LSU1 is from *Arabidopsis thaliana*. Explanation of the symbols: +++ good growth, ++ weak growth, + very weak growth, - no growth. The plasmids are described in **Suppl Table 2**.

		BD plasmids					
		pGBT9 (vector)	pJK1 (UP9C)	pJK11 (Joka2)	pJK6 (PB1)	pJK7 (ZZ)	pJK15 (LSU1)
AD plasmids	pGAD424 (vector)	-	-	-	-	-	-
	pJK2 (UP9C)	-	+++	-	-	++	+
	pJoka2 (Joka2)	-	+++	+++	+++	+	+++


A
pJK1
pGBT9
pJK2
pJoka2
pJoka8
pJoka20
SD-LT
SD-LTA
SD-LTH
+X-gal
B
2a
2b
8
20
2b
8
20
C-
C+


A
Pongo abelii
Pan troglodytes
Homo sapiens
Rattus norvegicus
Mus musculus
67
90
99
Macaca mulatta
51
100
Bos taurus
81
100
Ovis aries
79
Sorghum bicolor
Arabidopsis thaliana
Drosophila melanogaster
animals
100
100
Zea mays
100
52
Caenorhabditis elegans
99
100
Physcomitrella patents
39
Oryza sativa
35
51
Populus trichocarpa
Ricinus communis
Triticum aestivum
Vitis vinifera
Nicotiana tabacum
plants
B
PB1
LIR
NES
LIR
ZZ
LIR
LIR
UBA
LIR
N.tabacum
PB1
LIR
NES
LIR
ZZ
LIR
LIR
UBA
LIR
A.thaliana
PB1
ZZ
NES
LIR
KIR
UBA
H.sapiens
NLS
NLS

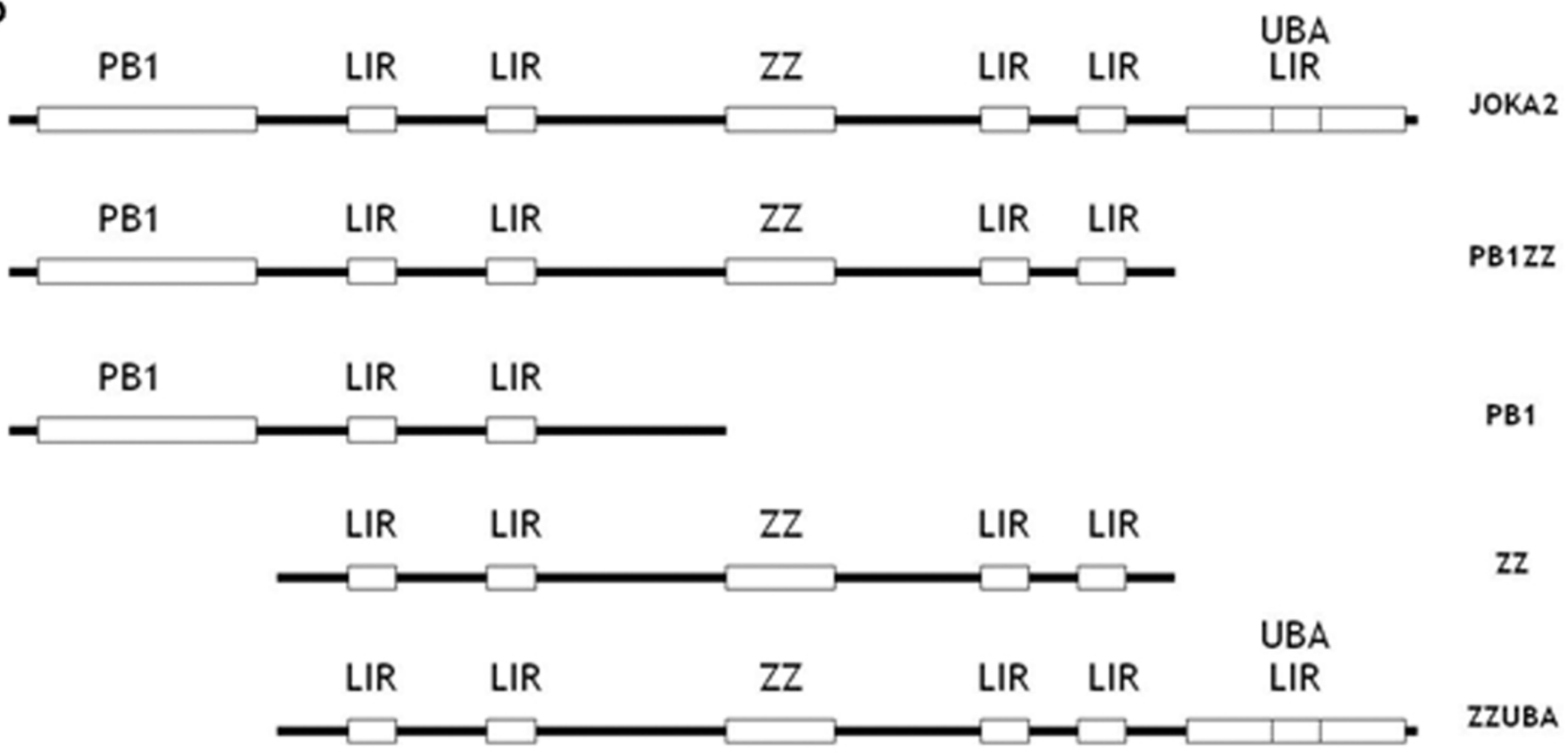
A

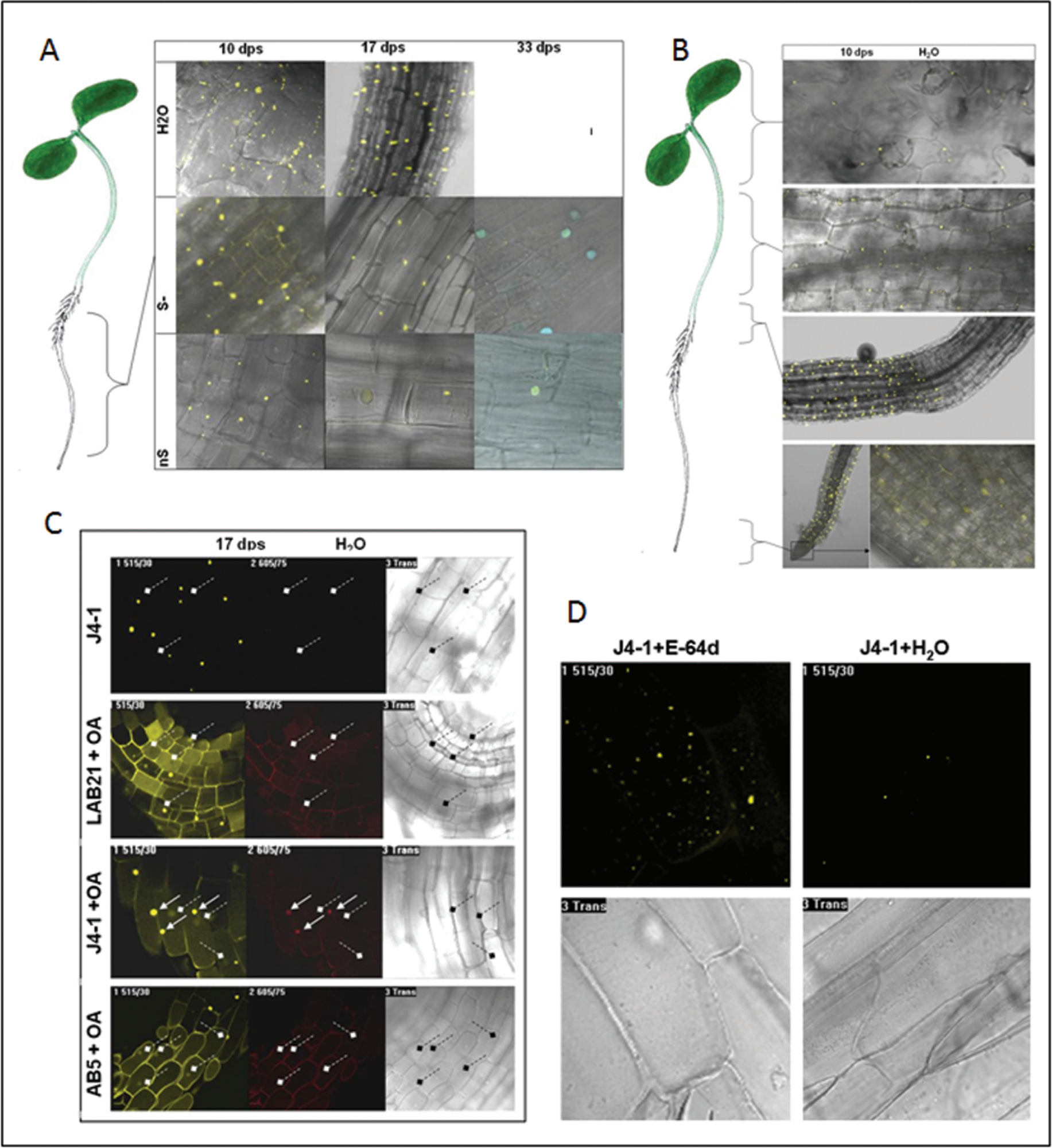


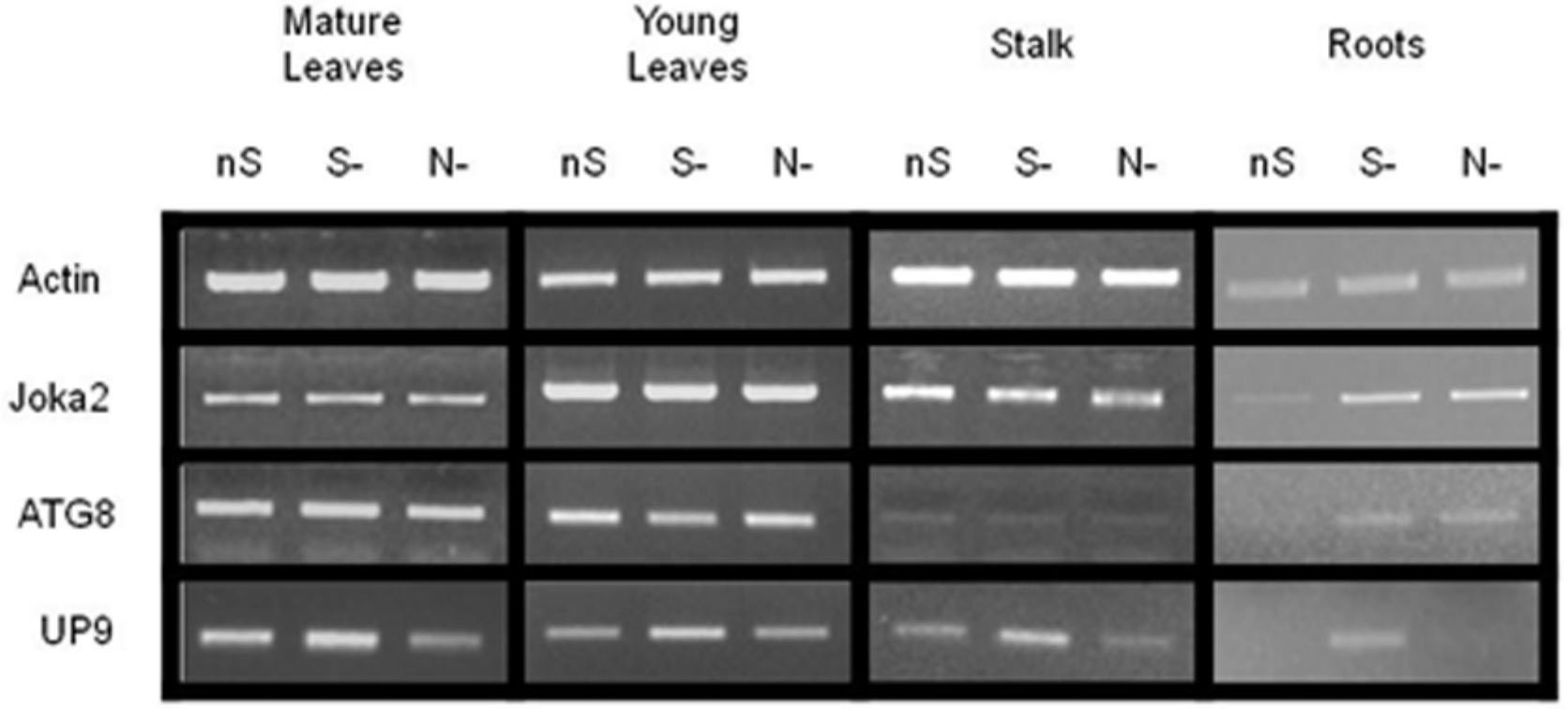
	AD	BD	growth
1.	ATG8	PB1	-
2.	ATG8	ZZ	-
3.	ATG8	ZZUBA	-
4.	ATG8	JOKA2	+
5.	PB1	ATG8	-
6.	ZZ	ATG8	-
7.	ZZUBA	ATG8	-
8.	PB1	PB1	-
9.	JOKA2	JOKA2	+
10.	Vector	PB1	+
11.	Vector	ZZ	-
12.	Vector	ZZUBA	-
13.	Vector	JOKA2	-
14.	PB1	Vector	-
15.	ZZ	Vector	-

	AD	BD	growth
16.	ZZUBA	Vector	-
17.	ATG8	Vector	-
18.	ZZUBA	ZZUBA	-
19.	PB1ZZ	ATG8	+
20.	ATG8	PB1ZZ	-
21.	JOKA2	ATG8	+
22.	Vector	ATG8	-
23.	JOKA2	Vector	-
24.	PB1ZZ	Vector	-
25.	PB1ZZ	PB1ZZ	+
26.	Vector	PB1ZZ	-
27.	Negative control		+
28.	Weak positive control		+
29.	Strong positive control		-

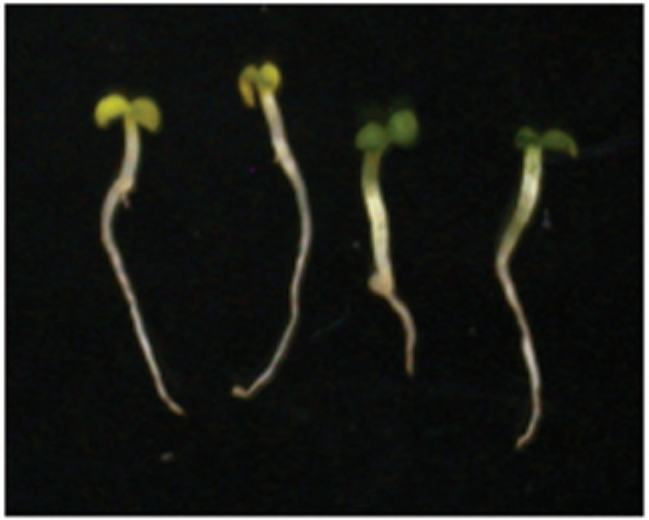
B




A
10 dps
17 dps
33 dps
H2O
S-
nS
B
10 dps
H2O
C
17 dps
H2O
J4-1
LAB21 + OA
J4-1 +OA
AB5 + OA
1 515/30
2 605/75
3 Trans
D
J4-1+E-64d
J4-1+H2O
1 515/30
3 Trans


Mature Leaves
Young Leaves
Stalk
Roots
nS
S-
N-
Actin
Joka2
ATG8
UP9

A



LAB21 AB5 J5-3 J4-1

B