Differential expression of IDA (INFLORESCENCE DEFICIENT IN ABSCISSION)-like genes in Nicotiana benthamiana during corolla abscission, stem growth and water stress

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Research article

Abstract

Background IDA (INFLORESCENCE DEFICIENT IN ABSCISSION)-like signaling peptides and the associated HAE (HAESA)-like family of receptor kinases were originally reported in the model plant Arabidopsis thaliana (Arabidopsis) to be involved in the regulation of abscission. IDA peptides, as cell-to-cell communication elements, appear to be implicated in many developmental processes that rely on cell separation events, and even in the responses to abiotic stresses. However, the knowledge related to the molecular machinery regulating abscission and other cell separation processes in economically important crops is scarce. In this work, we determined the conservation and phylogeny of the IDA-like and HAE-like gene families in relevant species of the Solanaceae family and analyzed the expression of these genes in Nicotiana benthamiana to identify members involved in abscission, stem growth and response to drought conditions. Results The phylogenetic relationships among the IDA-like members of the species of Solanaceae studied, grouped the NbenIDA1 (A and B) and NbenIDA2 (A and B) pairs of genes with the Arabidopsis prepropeptides related to abscission. In silico analyses of the regulatory elements of the promoter region of IDA-like members showed that these two pairs of genes, NbenIDA1 and NbenIDA2, contained drought and hormonal response elements, although NbenIDA2A lacked the hormonal regulatory elements. Expression analyses showed that both NbenIDA1 genes were upregulated during corolla abscission. NbenIDA1 and NbenIDA2 genes showed tissue differential expression under water stress conditions, since NbenIDA1 genes were highly expressed in stressed leaves while NbenIDA2 genes, especially NbenIDA2B, were highly expressed in stressed roots. In non-stressed active growing plants, nodes and internodes were the tissues with the highest expression levels of all members of the IDA-like family and their putative HAE-like receptors. Conclusion The results suggest that NbenIDA1A and NbenIDA1B are both involved in the natural process of corolla abscission while the NbenIDA1 and NbenIDA2 pair of genes are implicated in the response to water stress. The data also suggest that IDA peptides may be important during stem growth and development. These results add new evidence that the functional module formed by IDA peptides and its receptor kinases, as defined in Arabidopsis, may also be conserved in Solanaceae.

Background

The significance of the *INFLORESCE DEFICIENT IN ABSCISSION* (*IDA*)-like gene family is primary associated with the observation that *AtIDA* was deeply involved in the regulation of the abscission of floral organs and cauline leaves in *Arabidopsis thaliana* (Arabidopsis) [1–3]. Abscission is an active, organized and highly coordinated cell separation process allowing the detachment of entire vegetative and reproductive organs through the modification of cell-to-cell adhesion and breakdown of cell walls at specific sites on the plant body known as abscission zones (AZs), a discrete group of functionally specialized cells (for a review, see [4]). From an evolutionary point of view, abscission is a very favorable process that has several advantages such as seed dispersal as well as the shedding of no longer needed, damaged or infected organs. In addition, the abscission process is related to other processes such as senescence, pathogen defense and drought stress tolerance [5]. Abscission of aerial organs, on the other

hand, may become a major limiting factor of yield in an agricultural context. It is widely accepted that the control of abscission in Arabidopsis requires physical interaction of the hormonal peptide AtIDA, a pair of redundant receptor-like protein kinases, HAESA (HAE) and HAESA- LIKE2 (HSL2), and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) co-receptors (for a review, see [6]). This interaction activates a signal transduction through a downstream MAP kinase cascade that leads to the expression of multiple abscission-related hydrolytic enzymes such as pectin-methylesterases, polygalacturonases, cellulases, xyloglucan-endotransglycosylase/hydrolases and expansins [7, 8]. The release of this set of enzymes cause the disassembly of the cell wall and the dissolution of the middle lamella, resulting in the detachment of floral organs in Arabidopsis. Furthermore, in Arabidopsis *ida* mutants, petals remain indefinitely attached to the flower [1]. It has been also shown that synthetic IDA peptides were able to induce early floral abscission in Arabidopsis flowers [9].

The function of IDA peptides, as cell-to-cell communication elements, does not appear to be solely restricted to their roles in abscission. The activity of these peptides has also recently been involved in other developmental processes in Arabidopsis all of them settled on cell separation events such as the emergence of lateral roots and the root cap sloughing [10, 11]. In addition, IDA peptides have recently been involved in the response to abiotic stresses. Expression analysis of Arabidopsis *AtIDL6* and *AtIDL7*, for instance, revealed that these two genes are rapidly induced during various stress treatments [12]. It was subsequently determined experimentally that these peptides were involved in the stress response as modulators of reactive oxygen species (ROS) signaling [13]. Furthermore, treatments with AtIDL6 and AtIDL7 peptides caused downregulation of important stress-response key regulators like ZINC FINGER PROTEIN and WRKY transcription factors.

In addition to Arabidopsis, *IDA*-like genes have been identified in some crop species associated with organ abscission and the emergence of lateral roots [14] as well. Regarding organ abscission, particular members of the *IDA*-like gene family of tomato (*SlIDA1*), soybean (*GmIDA2a*), citrus (*CitIDA3*), litchi (*LcIDL1*), oil palm (*EgIDA5*) and yellow lupine (*LlIDA*) were highly expressed in leaf, flower or fruit abscission zones during abscission [15–19]. In addition, the *AtIDA* homologues of citrus (*CitIDA3*) and litchi (*LcIDA1*) were able to induce earlier floral organ abscission and to rescue the *ida2* abscission deficiency when ectopically expressed in Arabidopsis [16, 20]. All these data, together with the positive affect of treatments with synthetic IDA peptides on triggering organ abscission [9, 19, 21] strongly suggest conserved functions of *IDA*-like genes in regulating cell separation events during organ abscission.

The Solanaceae are an economically important family of flowering plants including several valuable agricultural crops, medicinal plants, spices, ornamentals and weeds. In these species, the abscission associated events such as those related to the detachment of flowers and fruits are of certain importance as in tomatoes, eggplant or peppers. However, the knowledge related to the molecular machinery regulating abscission in those species is very limited. In this work, firstly, we determined the conservation and phylogeny of the *IDA*-like and *HAE*-like gene families in relevant species of the Solanaceae and thereafter we analyzed the expression of these genes in *Nicotiana benthamiana* plants in order to identify

and discriminate members involved in organ abscission, stem growth and in the response to drought conditions. *N. benthamiana* is a model organism widely used in plant research. In this plant, the only organ that undergoes abscission is the corolla of the flower. The corolla abscission in *N. benthamiana* should be highly similar to that reported in *N. tabacum* [22]. The detachment of the corolla is due to the dissolution of the middle lamella and apparently to the disintegration of the parenchymal cells in its basal zone, a process that results in the detachment of the senescent corolla. Furthermore, the effect of water stress on the species of this contrasted genus has been the subject of major research and the physiological responses of these plants are also well known [23–26].

Results

The *IDA*-like gene family in the Solanaceae

Table 1 resumes a search on *IDA*-like gene families present in representative species of the *Nicotiana* genus such as *N. sylvestris*, *N. tomentosiformis*, *N. tabacum* and *N. benthamiana* in addition to other Solanaceae of agronomic interest such as tomato, potato, eggplant and pepper. All prepropeptides analyzed share two relevant characteristics, a signal peptide targeting the protein to the apoplast through the secretory pathway and a highly conserved PIP motif, typical of this gene family [12, 17].

The *IDA*-like families of the *Nicotiana* species *N. sylvestris* and *N. tomentosiformis* consisted of 5 members, while in *N. benthamiana* and *N. tabacum* these families are formed by 5 pairs of members, with one exception corresponding to the second member of the *NbenIDA4* pair that has not been found in the genomic screening. All *IDA*-like genes found in *Nicotiana* are new identifications, as the 6 members found in *S. melongena* and *C. annuum* and the 7 members of the *S. tuberosum* family. In *S. lycopersicum*, 5 out of the 8 IDA-like genes detected, members 1 to 5, were already described in [15] and named *SlIDA1-5*, while the other 3 peptides, *SlycIDA6-8*, are incorporated in the current work.

Phylogenetic relationship among IDA-like prepropeptides in Solanaceae

The phylogenetic relationships among the *IDA*-like members of the species of Solanaceae studied, in addition to those of Arabidopsis, are grouped in three major clades (Figure 1). Clade I is divided in two subclades, one of which includes exclusively the two Arabidopsis prepropeptides putatively related to abscission, AtIDA and AtIDL1 [1, 9]. The biggest subclade groups members of the all 8 Solanaceae species studied, as well as AtIDL8, the most divergent IDA-like peptide from Arabidopsis. In this subclade, Solanaceae members are further divided in two major groups. One of them (dark green in Figure 1) is characterized by the occurrence of SlycIDA1, a prepropeptide that has been experimentally associated with leaf abscission [15] and several members of the genus *Nicotiana* including NbenIDA1A and NbenIDA1B (the subject of this study, see below) and other prepropeptides such as NsylIDA1, NtomIDA1, NtabIDA1A and NtabIDA1B with promoter and gene sequences similar to those of NbenIDA1A and NbenIDA1B (Additional file 1). The other group includes other prepropeptides from the *Nicotiana*, *Solanum* and *Capsicum* genera (light green in Figure 1), with a small subdivision composed of AtIDL8 together with SlycIDA6, SlycIDA7 and StubIDA1 (lighter green in Figure 1). A second clade, clade II,

appears to be limited to the Solanaceae family. This clade includes prepropeptides from the *Nicotiana*, *Solanum* and *Capsicum* genera, but none from Arabidopsis, an observation that suggests that it might have diverged before the irruption of the Brassicaceae family 41 million years ago. The third clade, clade III, includes Arabidopsis IDA-like family members associated with processes different than cell separation, such as stress response [13]. The topology of the clade shows that there was a great diversification in Arabidopsis that generated at least 6 members, AtIDL2-7. It also includes prepropeptides from the *Nicotiana* and *Solanum* genera, but none from *Capsicum*.

It is worth mentioning that, of each pair of *N. tabacum* peptides (see above), there is one member of the pair closely related to the *N. sylvestris* peptide (member A), while the other one is more similar to that of *N. tomentosiformis* (member B). With one exception (*NbenIDA4B*), this correlation can also be observed for the two members of *N. benthamiana* peptides.

The EPIP motif of the IDA-like peptides in the Solanaceae

Sequence logo representations of the C-terminal part of IDA-like peptides (EPIP motif) corresponding to different alignments are presented in Figure 2. In general, there is a high level of conservation in most positions of the PIP motif (the 12 last residues of the protein) of the *IDA*-like families as observed across several species, belonging to different families such as Brassicaceae (Arabidopsis), Rutaceae (*Citrus*), Fabaceae (soybean and *Lupinus*), Sapindaceae (*Litchi*) and Solanaceae (*Solanum* and *Nicotiana*). An exception to this general rule is observed mostly in the AtIDL6-8 members of the IDA-like family of Arabidopsis (Figure 2B), that contain additional amino acids to the general R18H19N20 motif. These genes have been involved in processes other than cell separation, such as the responses to stress although some evidences in ROS challenged *idl7* and *idl6 idl7* double mutant suggest that they may be related to programmed cell death events [13]. In the Solanaceae, residues P11, S13A/G14P15S16, R/K18 and N20 are characteristic of the IDA-like peptides (Figure 2C) regardless of the process in which the peptides are apparently involved. On the other hand, the 10 initial residues of the EPIP motif in panel 2D that includes those members related to abscission, are rather variable. These observations suggest that in these sequences there is not a motif clearly associated with abscission.

Cis-acting regulatory elements in the promoter regions of the *N. benthamiana IDA*-like family

Figure 3 shows a schematic representation of the cis-acting regulatory elements along 1000 bp of the 5'-UTR region of the *IDA*-like family genes of *N. benthamiana* and *AtIDA* and *AtIDL1* of Arabidopsis. Searches for response elements to hormones related to ABA, methyl jasmonate (MeJa), AUXs or GAs, as well as response elements to biotic and abiotic stresses were performed. Interestingly, *NbenIDA1A* and *NbenIDA1B* present similar promoter regions containing response elements to ABA, MeJa and AUX as *AtIDA*, involved in the abscission process [1, 27, 28]. *NbenIDA1* and *NbenIDA2* gene pairs also carry drought response elements in their promoter regions. On the other hand, *NbenIDA2B*, *NbenIDA3A*, *NbenIDA4*, and the *NbenIDA5* pair are characterized by the occurrence of GA response elements (Figure 3).

Expression patterns of *IDA*-like and *HAE*-like genes in *Nicotiana benthamiana* during growth and abscission

Expression analysis of the family of *IDA*-like ligand peptides and their putative *HAE*-like receptors in different plant tissues of *N. benthamiana* are presented in Figure 4. *HAE*-like (*HSL*) receptors were identified through the analyses of the phylogenetic relationships between the *HAE*-like receptor-like kinases (RLKs) of Arabidopsis and Nicotiana (see Additional files 2 and 3). The selected plant material included different vegetative tissues of a plant in active growth (apical buds, young and mature leaves, nodes and internodes, and roots), as well as reproductive tissues (anthers, styles, stigmas, and fruits) at different developmental stages, including samples of the base of the flower corollas, a tissue responding in *N. benthamiana* to the abscission process. The data showed that all members of the *IDA*-like family of *N. benthamiana* were expressed to a greater or lesser extent in the tissues analyzed, except *NbenIDA2B*, which exhibited in comparison with the other members of the family, a reduced expression in most tissues. In general, *IDA*-like genes expression was the highest in nodes and internodes, although *NbenIDA1A* expression levels were not especially high in internodes. Interestingly, during the process of corolla abscission, expression of *NbenIDA1A* and *NbenIDA1B* at the base of the flower corolla increased with the stage of development of the tissue, in parallel to the progress of the abscission process (Figure 4B). Initially, *NbenIDA1A* presented a low expression level in stage 1, but these levels progressively increased until reaching very high expression in stage 5. Similarly, *NbenIDA1B* showed homogeneous basal expression levels in the first stages and reached maximum expression in stage 5, although at this stage these levels were lower than those detected in *NbenIDA1A*.

Expression of the RLKs belonging to the *HAE*-like family also was relatively high in most of the tissues analyzed. As observed for the *IDA*-like genes, the highest expressions of the putative receptors of the IDA-like peptides, *NbenHAE.1*, *NbenHAE.2*, *NbenHSL2.1* and *NbenHSL2.2*, were also registered in nodes, internodes and at the base of the corollas of the flowers during the last stages of development.

Expression patterns of *IDA*-like genes in *Nicotiana benthamiana* during water stress

The presence of drought response elements in the promoter regions of the *IDA*-like genes, e.g. *NbenIDA1A*, *NbenIDA1B*, *NbenIDA2A* and *NbenIDA2B* (Figure 3), suggested that the expression of these genes might be regulated by the water status of the plant. Therefore, we exposed actively growing plants of *N. benthamiana* to 6 (mild stress) and 8 (severe stress) days of water stress and the expression levels of all members of the *IDA*-like family in axillary buds, roots and leaves were determined (Figure 5). While no differences in gene expression were found in axillary buds (data not shown), levels of *NbenIDA1A* and *NbenIDA1B* dramatically increased in leaf blades of plants subjected to severe water stress. In contrast, this condition resulted in higher increases of *NbenIDA2A* and *NbenIDA2B* transcripts in roots, indicating differential roles of this gene family in response to water stress. Changes in the expression of the rest of genes were of minor relevance although it is worth to mention that these members tended to repress their expression levels in roots of plants subjected to water stress, although *NbenIDA5A* expression was also reduced in stressed leaves.

Discussion

IDA-like genes were searched in relevant genera of the Solanaceae family including several species of *Nicotiana* (*N. sylvestris*, *N. tomentosiformis*, *N. tabacum* and *N. benthamiana*), and other crops of agronomic interest such as tomato (*Solanum lycopersicum*), potato (*S. tuberosum*), eggplant (*S. melongena*) and pepper (*Capsicum annuum*) (Table 1). This gene family was present in all species analyzed [17] and as expected, their members contained a signal peptide targeting the protein to the apoplast through the secretory pathway and a conserved C-terminal part of IDA-like peptides, the PIP or extended (E)PIP motif (Additional file 4). The presence of this signal peptide in the sequence of all identified genes suggests a mechanism of posttranslational maturation in the apoplast similar to that described in Arabidopsis, where the prepropeptide is proteolytically processed by subtilisin-like serine proteinases to yield a bioactive peptide [29]. This cleavage that occurs between positions 6 and 7 of the EPIP motif [30], normally occupied by a K and a G respectively, leaves a bioactive peptide of 14 amino acids containing the PIP motif with G7 as N-terminal and N20 as C-terminal residues (Figure 2). It is accepted that the G7-N20 14mer constitutes the peptide signal controlling abscission in Arabidopsis [31] and that in the C-terminal part of many groups of this kind of hormonal peptides, the positions R/K18, H19 and N20 (Figure 2. A) constitute the recognition site for a co-receptor of the SERK family [32]. Thus, it has been proposed that in Arabidopsis the interaction of the IDA ligand peptide with HAE/HSL2 RLKs could lead to the recruitment of a co-receptor of the SERK family starting the signal transduction resulting in floral organ abscission [33]. The sequence logo of the EPIP motif of AtIDA and AtIDL1-5 (Figure 2, A) is very similar to those of the Solanaceae studied in here, since it holds the amino acids and characteristic domains described in Arabidopsis (Figure 2. C). Furthermore, the representation of the EPIP motif of all IDA peptides that have been associated so far with abscission, this is Arabidopsis AtIDA and AtIDL1 [1], citrus CitIDA3 [16], soybean GmIDA2a and GmIDA2b [15], *Litchi* LcIDL1 [20], oil palm EgIDA5 [17], *Lupinus* LlIIDA [19] and tomato SlycIDA1 [15] and its orthologs with very similar sequence detected in clade I of Figure 1, including StubIDA4, SmelIDA5, CaIDA4, NbenIDA1A and NbenIDA1B) suggests that the relevant positions of the EPIP motif are P11, S13A/G14P15S16 and R18H19N20 (Figure 2, D).

In spite of these similarities, there are other members of the *IDA*-like family in Arabidopsis (AtIDL6-8, Figure 2, B) recently associated with other processes than abscission, such as stress response [13]. For instance, position 14 in the Solanaceae is occupied by an A rather than a G, as in these members of Arabidopsis, although both are small nonpolar aliphatic amino acids that should allow similar binding conditions of the mature peptide with its receptor. However, the general H19N20 motif observed in the Solanaceae and in the Arabidopsis members implicated in abscission responsible of the recruitment of a SERK co-receptor, is not conserved in these sequences.

The high level of conservation of the EPIP motif and the reduced size of the mature peptide (an alignment of the complete coding sequences of these genes can be seen in Additional file 4), precluded the study of the phylogenetic relationships based on these premises and, therefore, a circular phylogenetic tree was generated using complete sequences encoding prepropeptides including a signal peptide and a variable region (Figure 1). This tree shows that the Arabidopsis *IDA*-like gene family exhibits a higher degree of

diversification than the Solanaceae genera studied, with the exception of *Nicotiana*. The two Arabidopsis prepropeptides putatively related to abscission, AtIDA and AtIDL1 [1, 27] nested in a small clade grouped with a more bigger clade including many *IDA*-like members from the different species of Solanaceae. This big clade was divided in two subgroups in one of which was nested the tomato SlycIDA1, associated with abscission [15], and its orthologs in *S. melongena*, *C. annuum*, *S. tuberosum* and in the four *Nicotiana* species studied. Interestingly, there were *IDA*-like paralogs genes in *N. tabacum,* named "A" and "B" members, that exhibited high similarity to the peptide pairs of *N. sylvestris* and *N. tomentosiformis*, respectively, supporting the hypothesis that *N. tabacum* (4.5 Gb, genome size) is an allotetraploid originated from the hybridization of *N. sylvestris* (2.6 Gb) and *N. tomentosiformis* (2.7 Gb) [34]. This explains why the *IDA*-like family in tobacco contains twice as many members than those of their parental organisms (Table 1). *N. benthamiana* is also an allotetraploid that shows "A" and "B" IDA members, except for the second member of the *NbenIDA4* pair that appears to be lost in this genome. The identification of *N. sylvestris* as one of the parental species of *N. benthamiana*, in contrast to *N. tabacum*, still needs additional confirmation [35].

The analyses of the cis-acting regulatory elements in the 5'-UTR regions of the *N. benthamiana IDA*-like family and Arabidopsis *AtIDA* and *AtIDL1* (Figure 3) failed to identify ethylene response elements, in agreement with the idea that *IDA*-like genes regulating the abscission process are not directly dependent upon ethylene [36, 37]. It is well known, however, that ethylene promotes both abscission and the expression of IDA-like genes, as shown in soybean, oil palm and citrus, for instance [15, 17, 38]. Based on this observation, it may therefore be estimated that *IDA*-like genes could act downstream of the ethylene signaling pathway as recently proposed by Meir and co-workers [39]. In contrast, the presence of response elements to AUXs, ABA, MeJa and GAs in the promoter regions of the genes examined was abundant, suggesting that these hormones may play a role in the regulation of the expression of these genes. The occurrence of functional indole-3-acetic acid (IAA) signaling in the abscission zone during organ separation, for instance, has been demonstrated by Basu and co-workers [40]. It has also been determined that ABA and MeJa have abscission-promoting effects, while the role of GAs is not entirely clear, since GAs appear to have different effects depending on the concentration and mode of application [6, 41]. However, it has been shown in citrus that flower pollination increased gibberellin A1 (GA_1) levels and reduced ovary abscission and that the treatment of unpollinated ovaries with GA_3 also suppressed ovary abscission [42, 43].

The analyses also indicated that the coding and promoter sequences of *NbenIDA1A* and *NbenIDA1B* are highly similar and that the hormonal regulatory elements of both promoters share the same response elements in similar positions, in addition to the same drought response element. Furthermore, the pair of *NbenIDA2* genes also contains drought stress response elements in their promoter regions (Figure 3). Likewise, the coding and promoter sequences of the *IDA1* genes in *N. attenuata*, *N. sylvestris, N. tomentosiformis, N. benthamiana* and *N. tabacum* are very similar and have the same response elements in the same positions in their promoters, except *N. attenuata* (Additional file 1).

The results described above (Figure 1, 2 and 3) suggested that in *N. benthamiana,* NbenIDA1A and NbenIDA1B peptides may be involved in the abscission process. This suggestion is also supported by the gene expression patterns found at the corolla base of the flowers during the process of natural abscission. Thus, Figure 4.B shows that there is a positive correlation between the expression of *NbenIDA1A* and *NbenIDA1B* and the developmental stage of the corollas of the flowers, the only tissue susceptible to abscission in this species. As described in citrus styles [44], the accumulation of the *CitIDA3* transcript increased with the progress of the abscission process, reaching the highest values during the late stages of development. The expression pattern of *NbenIDA1B* and especially of *NbenIDA1A* suggests that these genes are positive regulators of corolla abscission in *N. benthamiana.* Similarly, there seems to be a correlation between the expression of the *IDA*-like genes and that of their putative receptors of the *HAE*-like family, *NbenHAE.1, NbenHAE.2* and *NbenHSL.2.2* (Figure 4.C), that also increased during the last phases of the corolla abscission.

As described for *IDA*-like families in other species [12], the different members of the *N. benthamiana* family are also expressed in multiple plant tissues (Figure 4). This is not a surprise since the IDA-like signaling peptides, as cell-to-cell communication elements, function in several cell separation events, including lateral root emergence and root cap sloughing [10, 11]. Interestingly, in plants of *N. benthamiana* actively growing, the highest expression level of most members of the *IDA*-like family was found in nodes and internodes. It is worth mentioning that the promoter regions of *NbenIDA2B, NbenIDA3A, NbenIDA4, NbenIDA5A* and *NbenIDA5B* genes contain GAs response elements, and that these hormones are pivotal regulators of stem growth [45]. Moreover, all *HAE*-like genes analyzed also show higher expression levels in nodes and internodes, especially *NbenHSL2.1* and *NbenHSL2.2,* in parallel with the pattern observed for the *IDA*-like genes. These expression patterns might be linked to the formation of vascular bundles and to the cell elongation and division associated with the process of stem elongation implying cell wall remodeling. Thus, the results appear to suggest that the signaling module formed by the IDA-like peptides and its kinase receptors may be conserved in other plants such as *N. benthamiana*.

The occurrence of cis-acting elements related to the drought response in the gene pairs *NbenIDA1A-NbenIDA1B* and *NbenIDA2A-NbenIDA2B* (Figure 3) also suggested to test the response of the *IDA*-like genes to water stress conditions. In the experiment reported in Figure 5 it is clearly observed that the first pair of genes was highly expressed in leaves from *N. benthamina* plants severely stressed while in roots, the genes that responded to water deficit were the members of the second pair. Furthermore, these 4 genes are phylogenetically close to *AtIDA* and *AtIDL1,* two Arabidopsis genes that are induced under abiotic stress conditions [12].

Furthermore, *NbenIDA2B* shows basal or very low expression levels in all plant tissues analyzed (Figure 4. B), but its expression is highly increased in conditions of severe drought stress only in roots (Figure 5). Thus, this gene shows a very relevant tissue-specific differential expression, and although its function has not been elucidated in this work it can be suggested that might be related to the development of

lateral roots, since this is a common response to drought stress also attributed to IDA-like peptides [10, 14, 46].

It has been recently observed that IDA signaling peptides can certainly regulate important developmental processes as well as fundamental plant responses to environmental conditions [13]. Our data indicate that in the allotetraploid *N. benthamiana*, the *NbenIDA1* and *NbenIDA2* gene pairs are differentially involved in the responses to drought stress while only *NbenIDA1* genes are apparently implicated in the natural process of corolla abscission. These data suggest that IDA-like signaling peptides can play different biological roles in various tissues and under distinct abiotic conditions.

Conclusions

We have investigated the *IDA*-like and *HAE*-like gene families of different Solanaceae species, *S. lycopersicum, S. melongena, C. annuum,* S. *tuberosum,* and four species of the genus *Nicotiana, N. sylvestris, N. tomentosiformis, N. benthamiana,* and *N. tabacum* and determined their phylogenetic relationships. In the allotetraploid *N. benthamiana,* specific analyses of the EPIP motif and the cis-acting regulatory sequences and the examinations of the gene expression patterns of the *IDA*-like family have identified putative candidate *IDA* genes implicated in corolla abscission and in the response to water stress. The results suggest that the pair of genes *NbenIDA1A* and *NbenIDA1B* are both involved in the natural process of corolla abscission. Interestingly, *NbenIDA1* and *NbenIDA2* genes show specific differential expression under water stress conditions, since *NbenIDA1A* and *B* are highly expressed in stressed leaves while both *NbenIDA2* genes, especially *NbenIDA2B*, are highly expressed in stressed roots. In addition, nodes and internodes are the tissues with the highest expression of the *IDA*-like and *HAE*-like genes in normal active growing plants, suggesting that these peptides are also essential during stem growth and development. These results add new evidence that the functional module formed by IDA-like peptides and its receptor kinases as defined in Arabidopsis, may be conserved in Solanaceae.

Methods

Retrieval and sequence analysis

The EPIP motif of AtIDA (FGYLPKGVPIPPSAPSKRHNSFVNSLPH) was used to identify the *IDA*-like members of the selected Solanaceae species (*N. sylvestris, N. tomentosiformis, N. tabacum, N. benthamiana, Solanum lycopersicum, S. tuberosum, S. melongena and Capsicum annuum*) by tBLASTn and BLASTp inquiries in the Sol Genomics [47] web platform (https://solgenomics.net/tools/blast/), depending on the databases status. "N.sylvestris Genome", "N.tomentosiformis Genome", "N.tabacum BX Genome", "N.benthamiana v1.0.1", "Tomato ITAG release 3.20", "Potato PGSC DM v3 scaffolds", "Eggplant draft genome (release 2.5.1)" and "Capsicum annuum UCD10X genome chromosomes (v1.0)" databases [34, 48–52] were used, respectively. Arabidopsis AtHAE, AtHSL1 and AtHSL2 protein sequences were retrieved from Phytozome v12.1, TAIR10 database and were used to identify the *HAE*-like members of the selected Solanaceae species in the same way as described above. Newly identified genes

were named numerically, adding an "A", "B", ".1" or ".2" termination to the *IDA*-like or *HAE*-like gene pairs for the allotetraploids *N. tabacum* and *N. benthamiana*.

Sequence alignments were performed through MEGA7 software [53] using the ClustalW algorithm with default parameters (DNA Data Bank of Japan, DDBJ; http://clustalw.ddbj.nig.ac.jp/). Phylogenetic trees were created using the Neighbor-Joining method [54] using 1000 bootstrap replicates. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. The evolutionary distances were computed using the Poisson correction method [55] and are in the units of the number of amino acid substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

Peptide localization prediction and presence of signal peptides in the IDL amino acid sequences were analyzed using the TargetP [56] and SignalP-5.0 [57] services. Sequence logo representations were performed using WebLogo [58]. The Lesk color code [59] was used to highlight amino acid residues in sequences alignments and in sequence logo representations. Up to 1000 base pairs of promoter regions upstream of the start codon of the IDL genes of the *Nicotiana* species available in Sol Genomics databases were retrieved and submitted for cis-acting regulatory element analysis in PlantCARE [60]. Schematic representations of regulatory elements of the promoter sequences where created using IBS1.0.3 software [61].

Plant materials and growth conditions

N. benthamiana seeds were obtained from Dr. José Guerri and Dr. Karelia Velázquez of the Centro de Protección Vegetal y Biotecnología (IVIA, Moncada, Spain). The seeds were germinated on nutrient soil and transplanted individually in small pots with an artificial potting mix (50% vermiculite and 50% peat moss) in a plant growth chamber at 20/24 °C (night/day), 60% relative humidity and a 16/8-h light/dark regime. Water stress was induced by not watering the plants for 6 and 8 days for mild and severe stress conditions respectively.

RNA extraction and qPCR analysis

Basal portion of the corollas at different flower development stages as well as the rest of studied tissues were manually collected from the plants and frozen with liquid nitrogen. The tissue was grinded using Thomas Scientific's Liquid Nitrogen Cooled Mortar. Total RNA was extracted using Macherey-Nagel's NucleoSpin® RNA Plant, following the manufacturer's instructions. cDNA was synthesized from the RNA extraction using Thermo Fisher Scientific's SuperScript™ II Reverse Transcriptase, following the manufacturer's instructions.

Quantitative PCR analysis were performed using LightCycler® FastStart DNA MasterPLUS SYBR Green I reaction mix and a LightCycler 2.0 instrument (Roche, Basel, Switzerland) to determine the relative original transcript levels in each cDNA sample using gene-specific primers designed using Primer3Plus [62] and listed in Additional file 5. The fluorescence intensity data was obtained through LightCycler

Software version 4.1, and used to calculate the relative expression level of each gene through the ΔCt method using PP2A as a housekeeping gene [63]. In the water stress experiment, the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of IDL genes of *N. benthamiana* using watered plants and PP2A gene as controls, as described in [64]. Specificity of the amplification reactions was assessed by melting temperature profiling of the amplicons yielded by each primer pair.

Abbreviations

ABA: abscisic acid, AUX: auxins, GA: gibberellin, HAE: HAESA, IDA: INFLORESCENCE DEFICIENT IN ABSCISSION

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

Funding

Authors' contributions

DV, CD, MT and FRT designed the research, DV, CD, and FRT performed the research and analyzed the data, DV, MT and FRT wrote the article.

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Table

Table 1. *IDA*-like gene families in species of the Solanaceae family (genome localization from different Sol Genomics Network databases [47]). All prepropeptides are predicted to be localized in the secretory pathway according to TargetP [56] and SignalP-5.0 [57].

Gene name	Genome localization	Prepropeptide length (aa)	Predicted signal peptide length (aa)	PIP domain
NsylIDA1	Nsyl_KD945166.1:74265..74582 forward	105	39	PIPPSAPSKRHN
NsylIDA2	Nsyl_KD978144.1:88678..88980 reverse	100	32	PIPPSAPSKRHN
NsylIDA3	Nsyl_KD951180.1:40337..40579 forward	80	32	PIPPSAPSKRHN
NsylIDA4	Nsyl_KD977536.1:13349..13576 forward	75	22	PIPPSAPSQRHN
NsylIDA5	Nsyl_KD962079.1:38313..38564 forward	83	30	PIPASGPSRKHN
NtomIDA1	Ntom_KB972926.1:26032..26325 forward	97	37	PIPPSAPSKRHN
NtomIDA2	Ntom_KB954314.1:53025..53614 forward	96	32	PIPPSAPSKRHN
NtomIDA3	Ntom_KB969023.1:33965..34204 forward	79	31	PIPPSAPSKRHN
NtomIDA4	Ntom_KB956501.1:19193..19405 reverse	70	22	PIPPSAPSQRHN
NtomIDA5	Ntom_KB958630.1:30910..31161 reverse	83	30	PIPASGPSRKHN
NtabIDA1A	Ntab-BX_AWOK-SS18147:707412..707735 reverse	107	48	PIPPSAPSKRHN
NtabIDA1B	Ntab-BX_AWOK-SS9960:271769..272062 forward	97	37	PIPPSAPSKRHN
NtabIDA2A	Ntab-BX_AWOK-SS12153:24919..25221 reverse	100	32	PIPPSAPSKRHN
NtabIDA2B	Ntab-BX_AWOK-SS20685:67080..68370 reverse	96	32	PIPPSAPSKRHN
NtabIDA3A	Ntab-BX_AWOK-SS473:166199..166441 reverse	80	32	PIPPSAPSKRHN
NtabIDA3B	Ntab-BX_AWOK-SS2799:946688..946927 forward	79	31	PIPPSAPSKRHN
NtabIDA4A	Ntab-BX_AWOK-SS18001:26098..26325 forward	75	22	PIPPSAPSQRHN
NtabIDA4B	Ntab-BX_AWOK-SS12176:491822..492033 reverse	70	22	PIPPSAPSQRHN
NtabIDA5A	Ntab-BX_AWOK-SS18104:315608..315859 reverse	83	30	PIPASGPSRKHN
NtabIDA5B	Ntab-BX_AWOK-SS9524:125323..125574 forward	83	30	PIPASGPSRKHN
NbenIDA1A	Niben101Scf00570:62104..62373 reverse	90	36	PIPPSAPSK-----
NbenIDA1B	Niben101Scf01338:640730..641035 forward	101	35	PIPPSAPSKRHN
NbenIDA2A	Niben101Scf23219:7370..7663 reverse	97	32	PIPPSAPSKRHN
NbenIDA2B	Niben101Scf03368:114599..114892 reverse	97	32	PIPPSAPSKRHN
NbenIDA3A	Niben101Scf18667:206436..206678 forward	80	32	PIPPSAPSKRHN
NbenIDA3B	Niben101Scf01180:267334..267576 reverse	80	32	PIPPSAPSKRHN
NbenIDA4	Niben101Scf19133:87532..87771 forward	79	25	PIPPSAPSQRHN
NbenIDA5A	Niben101Scf03848:699324..699575 forward	83	30	PIPASGPSRKHN
NbenIDA5B	Niben101Scf02135:404883..405122 reverse	79	26	PIPASGPSRKHN
SlycIDA1	SL3.0ch05:4200134..4200439 forward	101	36	PIPPSAPSKRHN
SlycIDA2	SL3.0ch06:38623220..38623453 forward	77	30	PIPPSAPSKRHN
SlycIDA3	SL3.0ch04:5799910..5800149 forward	79	27	PIPPSSPSKRHN
SlycIDA4	SL3.0ch07:58068277..58068558 reverse	93	34	PIPPSAPSKRCN
SlycIDA5	SL3.0ch05:1629558..1629893 forward	111	29	LIPPSGPSRRHN
SlycIDA6	SL3.0ch09:540104..540379 reverse	91	26	PIPPSAPSCRSS
SlycIDA7	SL3.0ch09:546577..546855 reverse	92	27	PLPPSAPSCRSS
SlycIDA8	SL3.0ch11:533813..534061 reverse	82	28	PIPASGPSRKHN
StubIDA1	PGSC0003DMB000000071:252349..252627 reverse	92	27	PIPPSAPSCRSS
StubIDA2	PGSC0003DMB000000131:879373..879621 forward	82	28	PIPASGPSRKHN
StubIDA3	PGSC0003DMB000000243:905236..905523 forward	95	29	PVPPSGPSRRHN
StubIDA4	PGSC0003DMB000000410:16621..16935 reverse	104	36	PIPPSAPSKRHN
StubIDA5	PGSC0003DMB000000420:159814..160050 forward	78	26	PIPPSSPSKRHN
StubIDA6	PGSC0003DMB000000461:377302..377535 forward	77	30	PIPPSAPSKRHN
StubIDA7	PGSC0003DMB000000592:149451..149714 forward	87	34	PIPPSAPSERCN
SmelIDA1	Sme2.5_00993.1:18248..18481 forward	77	30	PIPPSAPSKRHN

SmeIIDA2	Sme2.5_04429.1:34294..34539 forward	81	28	PIPPSAPSLRHN
SmeIIDA3	Sme2.5_04724.1:40347..40592 forward	81	27	PIPASGPSRKHN
SmeIIDA4	Sme2.5_06686.1:19811..20078 forward	85	25	PIPPSAPSDRCN
SmeIIDA5	Sme2.5_08129.1:7336..7444 forward	102	34	PIPPSGPSKRHN
SmeIIDA6	Sme2.5_09763.1:10983..11228 reverse	81	26	PVPPSAPSDRCN
CaIDA1	PepperUCD10Xch04:178438292..178438525 forward	77	27	PIPPSAPSKRHN
CaIDA2	PepperUCD10Xch06:176812434..176812673 reverse	79	29	PIPPSAPSKRHN
CaIDA3	PepperUCD10Xch11:6480406..6480714 forward	102	33	PIPPSGPSKRHN
CaIDA4	PepperUCD10Xch11:4624209..4624499 forward	96	35	PIPPSAPSKRHN
CaIDA5	PepperUCD10Xch11:6480457..6480714 forward	85	18	PIPPSGPSKRHN
CaIDA6	PepperUCD10Xch11:27920042..27920356 reverse	104	24	PIPPSEPSPRHN

Additional Files

Additional file 1. Nicotiana *IDA1* promoters and coding sequences alignment.pdf. Alignment of the 5'-UTR sequences (500 bp) and the CDS of *NbenIDA1A*, *NbenIDA1B*, *NtabIDA1A*, *NtabIDA1B*, *NsylIDA1* and *NtomIDA1* genes. Start codon is highlighted in green, and cis-acting regulatory elements are highlighted as follows: brown line, abscisic acid; blue line, methyl jasmonate; red line, auxins; grey line, drought.

Additional file 2. HAE-like peptides phylogenetic tree.tif. Circular phylogenetic tree of HAE-like peptides of Arabidopsis thaliana and several species of the Solanaceae family (*N. benthamiana, N. tabacum, N. sylvestris, N. tomentosiformis, S. lycopersicum, S. tuberosum, S. melongena and C. annuum*).

Additional file 3. Solanaceae HSL family.pdf. *HAE*-like gene families in species of the Solanaceae family, (genome localization from different Sol Genomics Network databases [47].

Additional file 4. Alignment IDAs_ATH and Solanaceae_CINEMA Physicochemical.pdf. Sequence alignment of IDA-like peptides from several species of the Solanaceae family (*N. benthamiana, N. tabacum, N. sylvestris, N. tomentosiformis, S. lycopersicum, S. tuberosum, S. melongena and C. annuum*) and from Arabidopsis. The CINEMA color scheme is used to inform about the chemical nature of the amino acid residues in the EPIP domain (blue, polar positive; red, negative; green, neutral; white, non-polar aliphatic; purple, ocher and yellow, aromatic residues).

Additional file 5. qPCR primers.pdf. Primers used for quantitative PCR analysis

Figures

Figure 1

Unrooted circular phylogenetic tree of IDA-like prepropeptides of Arabidopsis thaliana and relevant species of the Solanaceae family such as N. Sylvestris, N. tomentosiformis, N. benthamiana, N. tabacum, S. lycopersiocum, S. melongena, C. annuum and S. tuberosum. Bootstrap values are shown in each node.
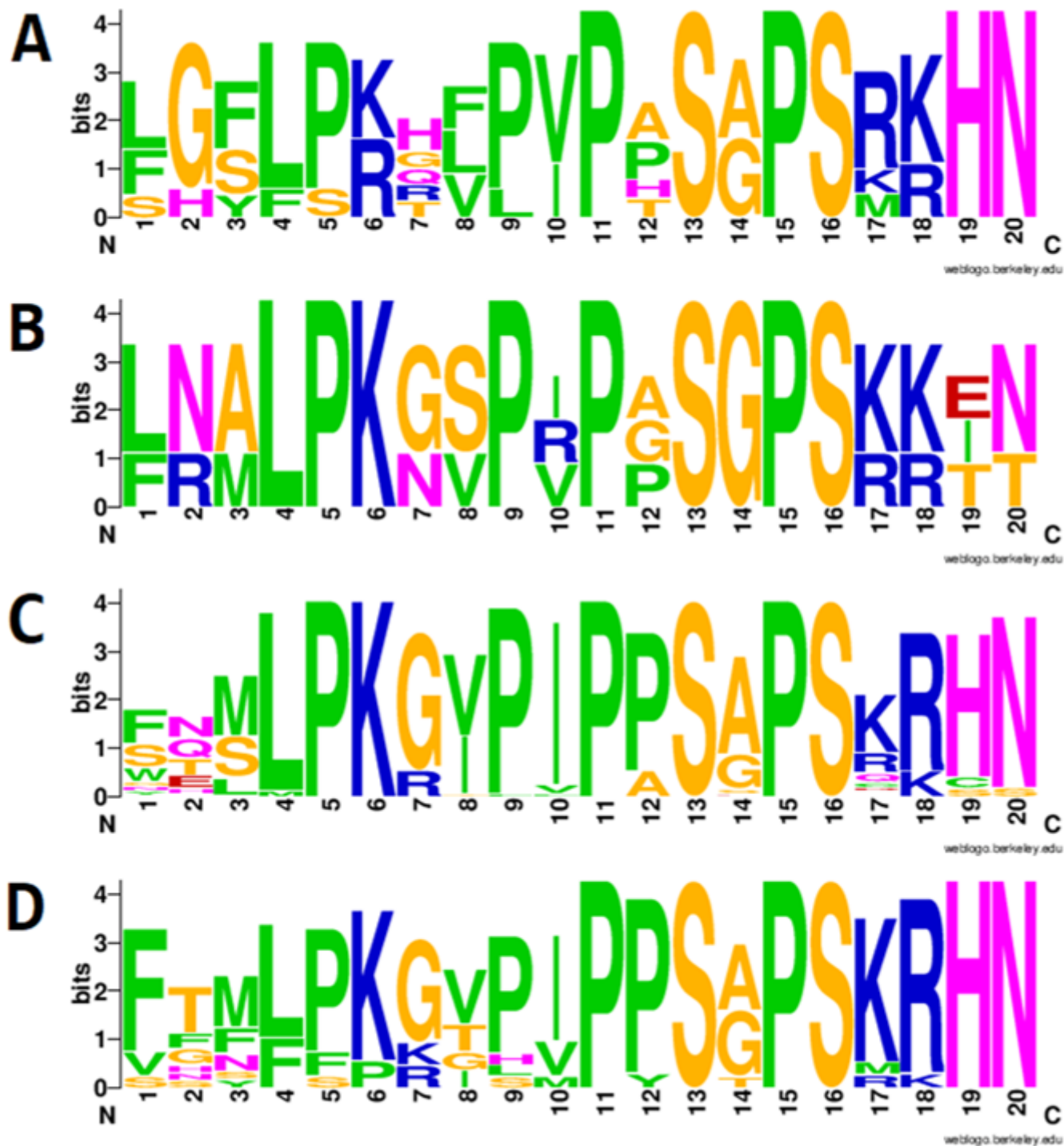
Figure 2

Sequence logo representation of the EPIP motif of the IDA-like peptides of Arabidopsis thaliana, (A) AtIDA and AtIDL1-5, and (B) AtIDA6-8, and (C) of the species of the Solanaceae family, N. Sylvestris, N. tomentosiformis, N. benthamiana, N. tabacum, S. lycopersiocum, S. melongena, C. annuum and S. tuberosum. (D) EPIP motif of IDL peptides previously associated with organ abscission such as AtIDA, AtIDL1, CitIDA3, GmIDA2a, GmIDA2b, LcIDL1, LlIDA, SlycIDA1 [1, 15, 16, 19, 20] and other peptides with very similar sequence including StubIDA4, SmelIDA5, CaIDA4, NbenIDA1A and NbenIDA1B.
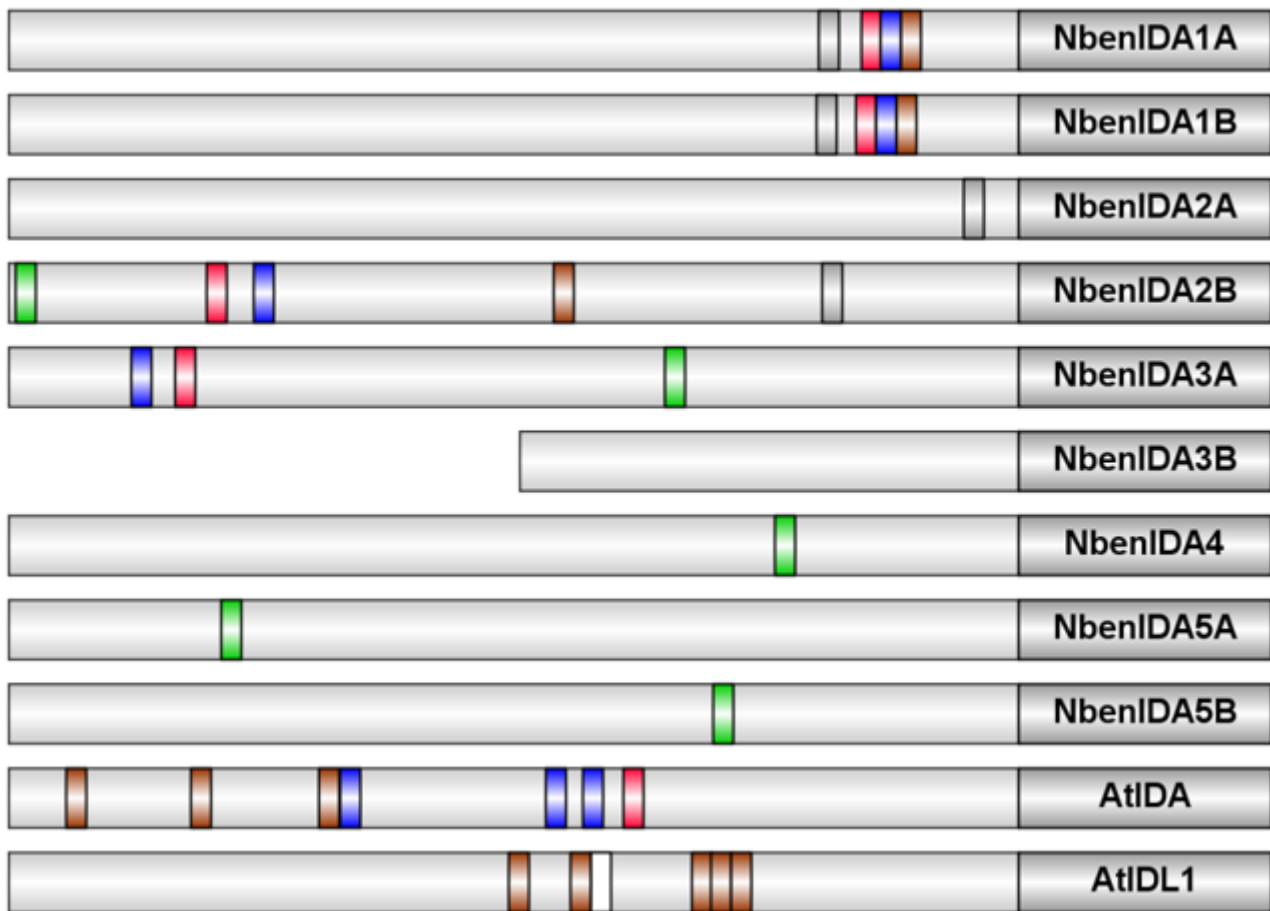
Figure 3

Schematic representation of cis-acting regulatory elements of the promoter regions of the N. benthamiana IDA-like gene family and Arabidopsis AtIDA and AtIDL1. Light grey boxes represent 1000 bp long promoter regions while dark grey boxes represent the 5' part of the gene. In-silico searches of response elements to hormones related to abscission such as abscisic acid, methyl jasmonate, auxins or gibberellins, as well as response elements to biotic and abiotic stresses were performed. Response elements color scheme: red, auxins; brown, abscisic acid; blue, methyl jasmonate; green, gibberelins; grey, drought stress; white, defense response. *NbenIDA3B promoter region is 493 bp long since the rest of the sequence is not available yet.
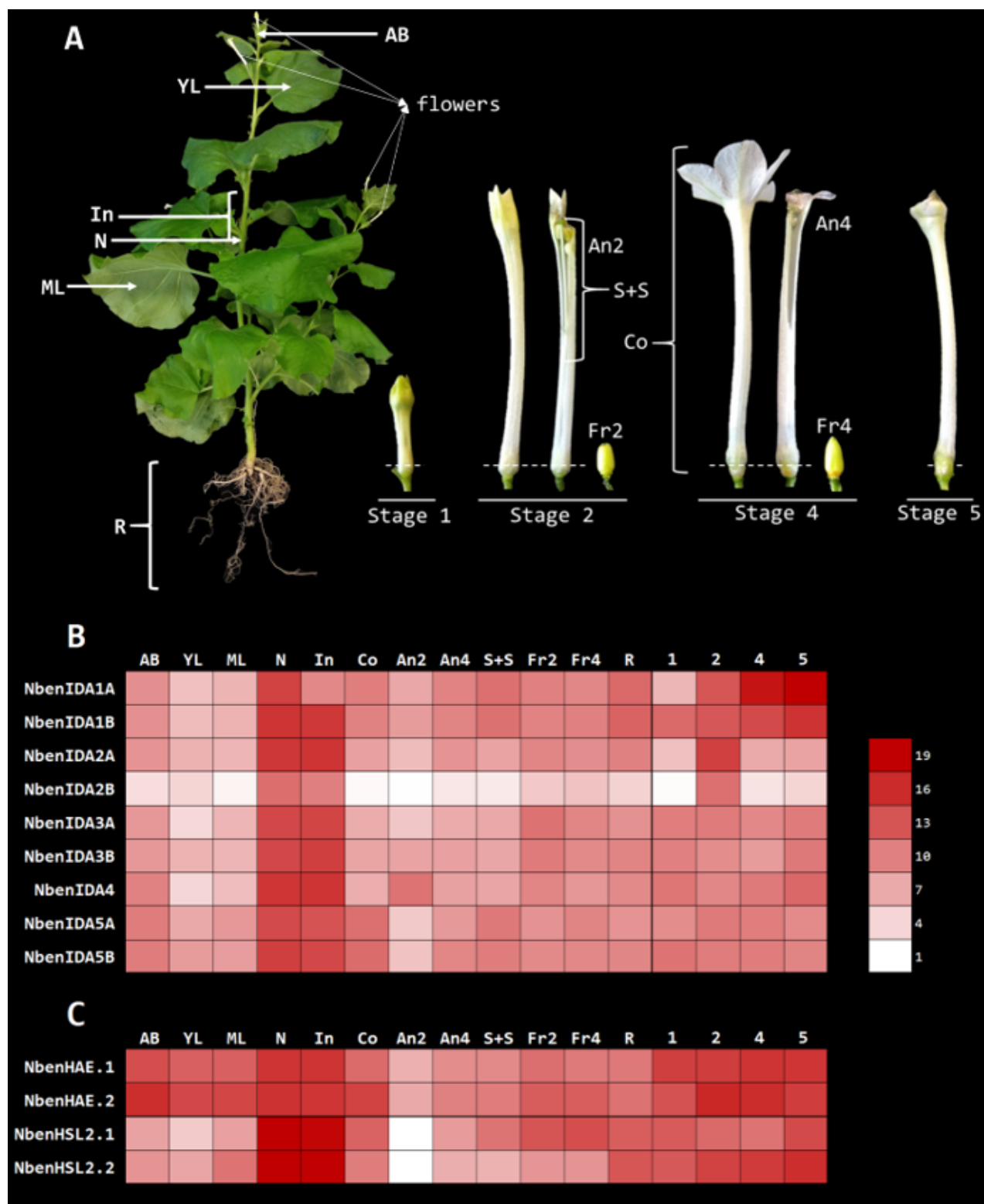
Figure 4

Expression patterns of IDA-like and HAE-like genes based on quantitative real-time PCR in several tissues of Nicotiana benthamiana at different developmental stages. (A) Floral organs (sepals were removed), fruits and vegetative tissues utilized for gene expression analysis. Dash lines mark the tissue collected from the base of the corolla. (B) Expression pattern of the IDA gene family in different plant tissues and in the base of the corolla at different stages. (C) Expression patterns of the HSL putative receptors of IDA

(NbenHAE and NbenHSL2) in different plant tissues and in the base of the corolla at different developmental stages. AB, apical bud; YL, young leaf; ML, mature leaf; N, node; IN, internode; CO, corolla; AN2, anthers, stage 2; AN4, anthers, stage 4; S+S, style and stigma; FR2, fruit, stage 2; FR4, fruit, stage 4; R, root; numbers 1 to 5, base of the corolla at 4 different stages. Expression levels relative to NbenPP2A are given next to the color scale column.
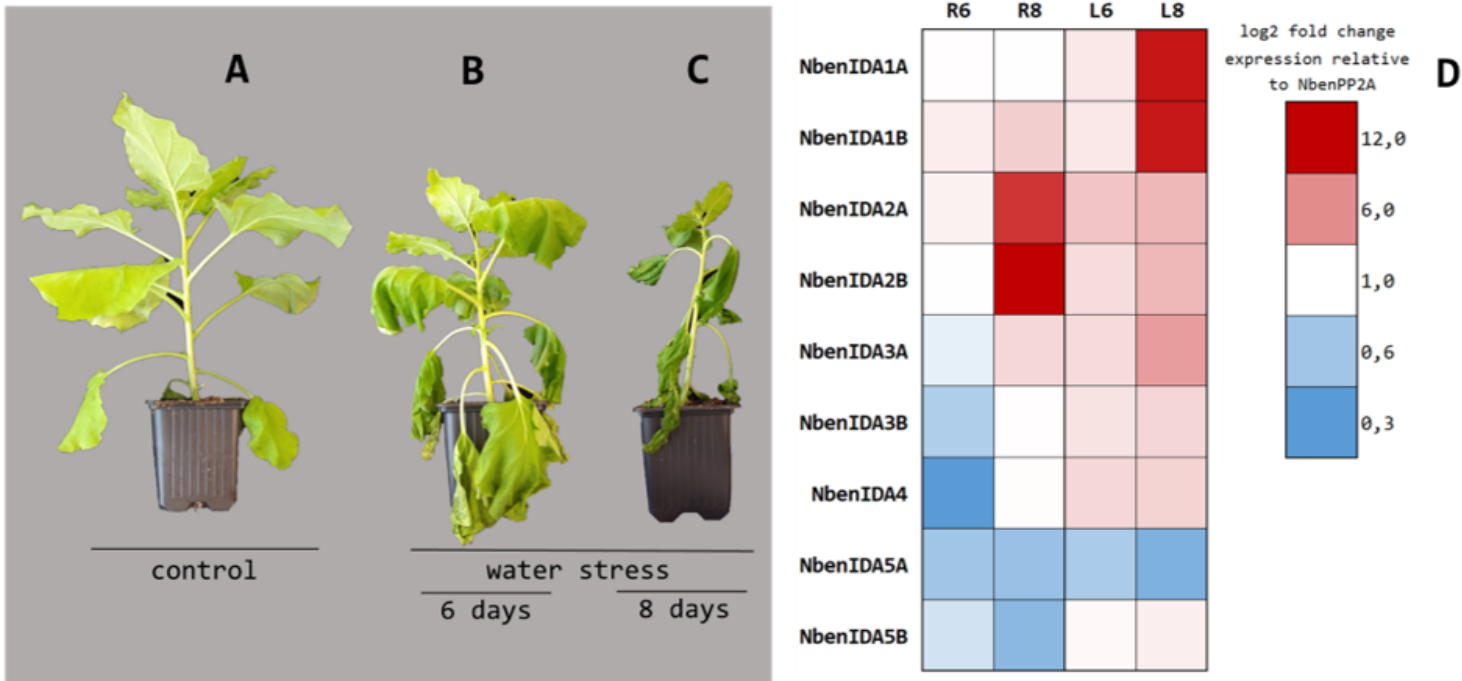

Figure 5

Expression patterns of IDA-like genes based on quantitative real-time PCR in control and water stressed plants of Nicotiana benthamiana. A) Control watered plant. B) Plant subjected to water stress during 6 days. C) Plant subjected to water stress during 8 days. D) Expression patterns in roots (R) and mature leaves (L) of water stressed plants during 6 or 8 days. Expression levels were calculated through the 2-ΔΔCT method relative to NbenPP2A gene expression in control plants and are next to the color scale column.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.pdf
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