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DELLA family duplication events lead to different selective constraints in angiosperms.

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Abstract

Gibberellic acid (GA) is a major plant hormone involved in several biological processes from the flowering to the symbiosis with microorganisms. Thus, the GA regulation is crucial for plant biology. This regulation occurs *via* the DELLA proteins that belong to the GRAS transcription factor family. DELLA proteins are characterised by a DELLA N-terminal and a GRAS C-terminal domains. It is well known that DELLA activity appears after the bryophytes divergence and then evolved in the vascular plant lineages. Here we present the phylogeny of DELLA across 75 species belonging to various lineages from algae, liverworts and angiosperms. Our study confirmed two main duplication events, the first occurring before the angiosperms divergence and the other specific to the eudicots lineage. Comparative analysis of DELLA subclades in angiosperms revealed the loss in Poaceae and strong alteration in other species of the DELLA functional domain in the DELLA2 clade. In addition, molecular evolution analysis suggests that each of the clades (named DELLA1.1, DELLA1.2 and DELLA2) evolved differently but copies of each subclade are under strong purifying selection. This also suggests that, although the DELLA functional domain is altered in DELLA2, DELLA2 orthologs are still functional and operate in a different way compared to DELLA1 copies. In angiosperms, additional duplication events occurred and led to duplicate copies in species, genus or family such as in the Fabaceae subfamily Papilionoideae. This duplication led to the formation of additional paralogs in the DELLA1.2 subclade (DELLA1.2.1 and DELLA1.2.2). Interestingly, both copies appeared to be under relaxing selection revealing different evolutionary fate of the DELLA duplicated copies.

Keywords: DELLA, evolutionary history, gene duplication, Fabaceae, molecular evolution

Introduction

Plant hormones play essential roles in various plant biology aspects and a huge diversity of these compounds have been identified so far (Davies 2010). Among them, the gibberellic acid (GA), a tetracyclic di-terpenoid molecule, is particularly involved in plant growth, seed germination and development or flowering (Gupta and Chakrabarty 2013). It also plays a role in responses to environmental stimuli (water, light, temperature) as well as during the symbiosis with microorganisms such as nitrogen-fixing bacteria and arbuscular mycorrhizal fungi (Lievens et al. 2005; Davies 2010; Ferguson et al. 2011; Gupta and Chakrabarty 2013; Takeda et al. 2015; Martín-Rodríguez et al. 2016)

Regulation of GA activity occurs through the DELLA (named according the conserved DELLA amino acid motif at the protein N-terminal domain) proteins which form a complex with the GA receptor GID1 repressing the expression of downstream GA-responsive genes (Ueguchi-Tanaka et al. 2007; Sun 2011). Fixation of GA to the GID1 receptor induces the proteasome-dependant degradation of DELLA proteins (Fu et al. 2002). This GA-GID1-DELLA complex has been observed in all the investigated vascular plants but is absent from bryophytes although a DELLA copy has been identified in *Physcomitrella patens* (Yasumura et al. 2007; Sun 2011). This suggests that the DELLA repressor activity of GA evolved after the bryophyte divergence (~430 MYA) and there is no functional data about the DELLA role and mode of action prior this divergence (Kenrick and Crane 1997; Yasumura et al. 2007; Wang and Deng 2014). In angiosperms, DELLA proteins are known to be involved in the regulation of a wide range of fundamental biological processes such as flowering, root and shoot development, and is a key regulator of both arbuscular mycorrhizal and root nodule nitrogen-fixing symbiosis (Maekawa et al. 2009; Ferguson et al. 2011; Floss et al. 2013; Yu et al. 2014; Pimprikar et al. 2016). For example, during nitrogen-fixing symbiosis, DELLA is thought to be a key regulator of the infection process by directly regulating symbiotic genes *NSP1/NSP2*, *ERN1*, *NF-YA1* and by enhancing the binding between CYCLOPS and NSP2 (Fonouni-Farde et al. 2016; Jin et al. 2016). Recently, DELLA proteins were also shown to be likely involved in the cytokinin genetic pathway regulation during nodulation in *Pisum sativum* L. (Dolgikh et al. 2019).

DELLA constitutes a subfamily of the GRAS transcription factors superfamily and DELLA proteins are characterised by a N- and C- terminal conserved DELLA and GRAS domains respectively (Pysh et al. 1999). The GRAS family encompasses between 8 and 13 subfamilies according to authors and surveyed species (Cenci and Rouard 2017). Although evolutionary history of the GRAS family has been recently investigated in representative angiosperms model species (Cenci and Rouard 2017), there is little data about the DELLAs evolution in angiosperms.

In this context, we performed a large screening of DELLA proteins in more than 50 species representing all the main plant orders. This allowed us to confirm different duplication events in the DELLA subfamily, with a subclade exhibiting severe alterations of the DELLA functional domain. In addition, molecular evolution analysis revealed that the angiosperms DELLA subclades have experienced different selective constraints with currently a strong purifying constraint within each of these subclades.

Material & Methods

Data mining

Proteomes of 75 plant species covering the main Angiosperms lineages (list available in Supplementary Table S1) were screened against the Pfam database (Finn et al. 2016) and hits with an e-value lower than 0.01 and both GRAS (PF12041.8) and DELLA (PF03514.14) domains were retained. To complement this search and take into account potential missing domains (in cases of losses or miss-annotated proteins), published and well characterized DELLA protein from *Arabidopsis thaliana*, *Oryza sativa*, *Lupinus angustifolius*, *Medicago truncatula* and *Amborella trichopoda* (Sato et al. 2014; Cenci and Rouard 2017; Hane et al. 2017) were used as queries for a tBLASTn v2.7.1+ search against the 75 plant species with default parameters (Camacho et al. 2009) and an e-value threshold of 1^{-60} . Finally, protein sequences shorter than 200 amino acids were removed from the dataset to eliminate partial assembly and putative remnants (retained and removed sequences are available in Supplementary Data S1 and S2 respectively).

In order to explore the absence of DELLA proteins in algae, the Algae-PrAS database containing 31 genomes (<http://alga-pras.riken.jp/>) was also screened with the reference DELLA sequences from *A. thaliana* and *O. sativa* as queries using the BLASTp v2.7.1+ algorithm and an e-value threshold of 0.01. Until recently, scarce data were available for Zygnematophyceae algae, the likely closest lineage of land plants. Two Zygnematophyceae genomes (*Mesotaenium endlicheranum* and *Spirogloea muscicola*, this latter belonging to a new subclass likely to be the closest from the Zygnematophyceae/embryophytes separation) have been published and were screened for DELLA homologs (Cheng et al. 2019).

Phylogenetic analysis

Retained protein sequences were aligned using MUSCLE (Edgar 2004) with default parameters. Alignment was subjected to maximum likelihood analysis using IQ-TREE v1.5.5 (Nguyen et al. 2015). The best-fitted evolutionary model was determined using ModelFinder as implemented in IQ-TREE (Kalyaanamoorthy et al. 2017). Maximum likelihood analysis was conducted with 10,000 replicates Ultra Fast Bootstraps (Hoang et al. 2018). Trees were visualized and annotated using the Interactive Tree of Life platform (Letunic and Bork 2016).

Molecular evolution analyses

For each clade of interest identified through the phylogenetic analysis, sequence logos were generated from protein alignments using the WebLogo v3.5 platform with default parameters (Crooks 2004). In addition, to compare signature of selection among DELLA homolog clades identified in phylogeny, we estimated dN/dS ratios from all pairwise comparison of protein-coding sequences using *yn00* module from the PAML package v4.9g (Yang and Nielsen 2000; Yang 2007). We also used the RELAX hypothesis testing framework from HyPhy (Pond et al. 2005; Wertheim et al. 2015) to look for relaxation ($K < 1$) or intensification ($K > 1$) of the signature of selection. To estimate signature of selection, codons were aligned according to the protein alignment using the pal2nal v14 Perl script (Suyama et al. 2006) and all gapped positions were removed. We

used the following command line for each clade tested (SupplementaryTable2): *hyphy relax CPU=1 --alignment input_file.fna --code Universal --type Nucleotide --keyword value Foreground*.

Results and discussion

DELLA family emerged with land plants and experienced multiple duplication events

To understand the origin of the DELLA family, the Algae-PrAS database that contains more than 30 genomes of non-land plants species (green and red algae, Glaucophyceae) and other organisms such as Oomycetes and diatoms was mined using DELLA protein sequences from *Arabidopsis thaliana* and *Oryza sativa*. Results confirmed the absence of DELLA orthologs in these species. In the two Zygnematophyceae genomes, no DELLA were detected but other GRAS family members were identified and are likely resulting from horizontal gene transfer from bacteria (Cheng et al. 2019). These findings are consistent with results from Yasumura et al. (2007) who suggested an emergence of DELLA proteins along with the land colonization by plants. To improve our understanding of the evolutionary dynamics of DELLA family, 75 land plants genomes were investigated and 293 DELLA orthologs were identified by the phylogenetic analysis (excluding putative remnants and misassembled sequences). Among them, one sequence with a DELLA functional domain was identified in the liverwort *Marchantia polymorpha* (Marpol_Mapoly0058s0044.1) consistently with the hypothesis that the DELLA family originated at least in the common ancestor of the vascular and non-vascular plants (Hernández-García et al. 2019). Previous findings revealed that the GID1-DELLA complex does not exist in the bryophytes lineage (Yasumura et al. 2007; Hirano et al. 2007). In bryophytes, DELLA has been recently proposed as transcriptional co-activator that could have been exapted in vascular plants (Hernández-García et al. 2019). Previously published results based on RNAseq data and putative DELLA's interactors in *Physcomitrella patens* (no DELLA-GID1 interaction), *Solanum lycopersicum* and *Arabidopsis thaliana* suggest that DELLA evolution is correlated with an increasing coordination of transcriptional programs between bryophytes and vascular plants (Briones-Moreno et al. 2017).

Phylogenetic analysis shows that DELLA family underwent multiple duplication events at different points of the land plant evolution as recently shown by Hernández-García and colleagues (2019). Indeed, results revealed a first duplication event that occurred before the divergence of angiosperms (Fig.1, with branch support values to be retrieved in the Supplementary Data S3) leading to the formation of two main clades, called DELLA2 (blue clade in Fig.1; corresponding to DELLA3 in Hernández-García et al. 2019) and DELLA1 (orange, green and red clades) accordingly to Cenci and Rouard (2017). DELLA sequences from *Marchantia polymorpha* and the lycophyte *Selaginella moellendorffii* seem to belong to the DELLA2 clade; however, these branches are not supported (UFB value < 50%) and these sequences are likely at the base of the whole DELLA family. As demonstrated in recent phylogeny and in this study the DELLA1/DELLA2 clades emergence is probably due to the Whole Genome Duplication (WGD) event that occurred prior the seed plants (c.a 319 MYA) (Jiao et al. 2011; Hernández-García et al. 2019). Another level of duplication has

been identified in the DELLA1 clade. Indeed, this latter, with the basal Angiosperms *Amborella trichopoda* at its base, can be divided into three main subclades (Fig.1). The first, in green, is exclusively composed by Monocot species whereas the two others only contain Eudicot species. These two Eudicot subclades emerged from the core Eudicot WGD event that occurred approximately 125 MYA (Van de Peer et al. 2017) and were arbitrarily named DELLA1.1 (orange clade in Fig.1) and DELLA1.2 (red clade). Finally, additional duplication events limited to species or subclades were identified such as in Brassicaceae DELLA1.1 or the DELLA1.2 of Papilionoideae (WGD of the Papilionoideae common ancestor, around 60 MYA, see below).

For each sequence, gene structure was extrapolated from the annotation file, when available, and results were mapped on the phylogeny (Fig. 1; outer circle). Excepting few sequences, most of the genes present a non-intronic structure and a length ranked between 1000 and 2000 bp.

Analysis of the functional domains revealed alterations in DELLA2

DELLA proteins are characterised by the presence of two functional domains. The first (DELLA) is in the N-terminal region of the protein and is specific to the DELLA family whereas the second (GRAS) is shared with other members of the GRAS superfamily. Functional analysis of the DELLA domain in the model plant *Arabidopsis thaliana* characterised two motifs required for the interaction with GID1 (Murase et al. 2008): DELLA\$LYXVXXXXMAXVAXXLEXLEX\$ and TVHYNPXXLXXWXXXM (where X and \$ stand for any and non-polar amino acids respectively).

DELLA and GRAS functional domains identified using the Pfam search were mapped on the phylogenetic tree (Fig.1, second outer circle) and revealed that most of the sequences possess both domains with few exceptions that can be explained by assembly and annotation issues or putative pseudogenes. However, it appears that all the six Poaceae from the DELLA2 clade have lost the DELLA functional domain (absence of red right pointing pentagram in Fig.1, and Supplementary Figure 1). The loss of the DELLA domain has been previously reported in rice (Itoh et al. 2005; Cenci and Rouard 2017) and our results indicate that this loss is likely to have occurred before diversification of the Poaceae from their common ancestor. Indeed, Monocots from other orders such as *Spirodela polyrhiza*, *Zostera marina* (two Alismatales) and *Musa accuminata* (Zingiberales) retained both DELLA and GRAS domains (Supplementary Figure 1). At the opposite, all Monocots DELLA1 proteins have a full DELLA domain. Previous functional analysis of the two DELLA2 copies of rice (also called SLRL1 and SLRL2 for SLR-like1 and 2) revealed that SLRL1 is positively affected by GA treatment whereas SLRL2 is not. In addition, trans-complementation of *slr1* (DELLA1) mutant by SLRL1 rescued the phenotype (Itoh et al. 2005). Since the N-terminal DELLA domain is missing in SLRL1 and 2, proteins were not degraded following the GA treatment but also exhibit a lower activity against GA than a SLR1 truncated copy missing the DELLA domain (Itoh et al. 2005). Itoh and colleagues suggest that SLR1 and SLRL1 can function together and are required for a fine-tuning of GA signalling.

In a second approach, the focus has been made on the DELLA functional domain extracted from the protein alignment and amino acids conservation represented using the WebLogo tool (Crooks 2004) for each main

clade of DELLA (Fig.2). Results revealed a high conservation of amino acids required for interaction with GID1 across the different DELLA1 subclades (DELLA1.1, DELLA1.2 and DELLA1 from Monocots). However, severe disruption of the DELLA motifs can be observed in the DELLA2 clade (Fig.2; red arrows). Indeed, the methionine from the MAXVA motif and at the end of the second DELLA subdomains is not conserved anymore among the DELLA2 sequences. Other disruptions of the DELLA domain are also characteristic of the DELLA2 clade such as the low conservation of the first leucine in the DELLA first motif or the alteration of the TV motif in the second DELLA motif (Fig.2). This low-conservation pattern observed in the DELLA2 clade could be explained by different hypothesis such as the pseudogenisation of the DELLA2 copies, a new function of these proteins, or another way for these proteins to interact with GID1.

To test whether the DELLA2 clade is under relaxed selective constraints, different evolutionary tests were applied. To this aim, only sequences with entire DELLA domains were retained and evolutionary tests performed on the domain alignment. The analysis of non-synonymous *versus* synonymous (dN/dS) revealed a higher dN/dS ratio when comparing DELLA2 to the other DELLA1 subclades, whereas these latter had similar dN/dS ratios (Fig.3). To determine if relaxed selection is acting on the DELLA2 clade, a branch model (RELAX) was applied with the DELLA2 clade marked as foreground branch (Supplementary Table 2). According to pairwise comparisons, estimating the selective intensity parameter (K) suggests that the DELLA2 clade is subjected to an ongoing process of relaxing selection ($K=0.81$, $p\text{-val}\approx 0.00074$, $LR=11.39$). Conversely, the higher value found for DELLA1 ($K=1.28$, $p\text{-val}\approx 0.00038$, $LR=12.61$) suggests intensification of the purifying selection ($K>1$) to maintain the proper role of DELLA1. In addition, logo and amino acid frequency for each position of the DELLA motifs (Fig.2 and Supplementary Table S3) show that mutations of essential amino acid in DELLA2 clade are shared by most of the sequences in this clade. For example, methionine from MAXVA stretch is replaced by leucine in 100% of sequences. Similarly, almost 90% of DELLA2 sequences share a glycine instead of a glutamate normally expected at the second position of the DELLA first motif. Altogether, these analyses suggest that modification of DELLA2 sequences occurred in the DELLA2 ancestor and then, have been maintained. These results are in line with a previous report suggesting that positive selection acted on the DELLA family during its evolution (Chen et al. 2013). To date, there is no detailed studies of DELLA2 proteins from Eudicot lineage. However, it is possible that, similarly to Poaceae DELLA2, these proteins function along with their DELLA1 counterparts and act as weaker GA signalling repressors without being degraded. Interestingly, it has been shown that the RGL1 protein of *Arabidopsis thaliana* (DELLA1.2 subclade) works without being degraded upon GA application (Wen and Chang 2002). All together, these results suggest that the multiple rounds of duplication in the DELLA family led to a slight subfunctionalization of DELLA copies, all involved in the GA regulation pathway. In their work, Yasumura and colleagues (2007), proposed a stepwise evolution of the DELLA-GA complex in plants with the GID1-DELLA interaction evolving in vascular plants. Here, we show that the DELLA family pursued its evolution in Angiosperms by experiencing several rounds of duplication, leading to sub-clades under different selective constraint. In addition, our results revealed that the loss of DELLA domain is shared among the DELLA2 proteins of Poaceae, supporting the hypothesis of a different mechanism (degradation-independent for example) for DELLA in this family (Itoh et al. 2005).

DELLAs from the Fabaceae show similar evolutionary pattern compared to that of the DELLA family

DELLA proteins play major role in various biological aspects of plant life. In legumes, they also have a critical role for the establishment of the root nodule nitrogen-fixing symbiosis. Recently, DELLA proteins have been proposed to be also involved in the regulation of cytokinin genetic pathway through interaction with KNOX and BELL transcription factors (Dolgikh et al. 2019). Thus, a focused analysis on the DELLA sequences from the Fabaceae family has been performed and showed similar evolutionary pattern compared to results discussed above.

First, the evolutionary pattern of DELLAs confirmed that the three main DELLA clades (DELLA2, DELLA1.1 and DELLA1.2) are conserved among the Fabaceae (Fig.4A). DELLA1 and DELLA2 copies both present mutations in amino acids involved in the interaction with GID1 (Fig.4B). The branch model analysis on both clades showed that DELLA1 and DELLA2 copies have a similar pattern of selection with no sign of relaxation/intensification (Supplementary Table S2). However, no change in the selective constraint was detected among the different clades suggesting that, also in Papilionoideae, there is no positive selection acting on them.

Second, in the DELLA1.2 subclade, an additional duplication event occurred leading to the formation of two subclades arbitrarily called DELLA1.2.1 and DELLA1.2.2. These two subclades likely originated from the Papilionoideae WGD that occurred 60MYA. Analysis of the selective constraints strongly suggests that both paralogs ($K=0.25$, $p\text{-val}=7.3\text{E-}08$, $\text{LR}=28.98$ and $K=0.26$, $p\text{-val}=7.2\text{E-}08$, $\text{LR}=29$ for DELLA1.2.1 and DELLA1.2.2 respectively) are under pseudogenization process. This is also supported by the apparent loss of DELLA1.2.1 copy in seven out of the 15 legume samples investigated.

Conclusion

DELLA proteins have a critical role in numerous aspects of plant life, from development to flowering but also in specific aspects such as in the nitrogen-fixing symbiosis. Phylogenetic analysis of DELLA family including more than 70 species confirmed its emergence in land plants along with its absence in algae. Results of phylogenetic analysis of the DELLA family is consistent with previous reports and show the existence of two main duplication events leading to the formation of three clades: DELLA1 and DELLA2 clades emerged from a duplication event that occurred before the Angiosperms divergence and two additional DELLA1 subclades (DELLA1.1 and DELLA1.2) emerged following the Eudicots whole genome duplication. A detailed analysis of the evolution of the three DELLA clades revealed that they are all under purifying selection although their ancestors seem to have been subjected to a relaxed selective constraint. In the DELLA2 clade, our results strongly support the loss of the DELLA domain before diversification of Poaceae and severe alterations of this motif in other DELLA2 proteins suggesting that DELLA2 protein could function in a degradation-independent manner. Finally, DELLA proteins from the Fabaceae family revealed a similar evolutionary history with additional duplication due to the Whole Genome Duplication of the Papilionoideae

ancestor leading to additional DELLA1.2.1 and DELLA1.2.2 clades. Both clades are under relaxing selection suggesting they are likely to be pseudogenized or neofunctionalized.

All together this study supports the hypothesis of a complex evolutionary history of DELLA proteins in Angiosperms with multiple changes in the selective constraint. To extend these results, functional data on the different DELLA copies from both Monocots and Eudicots would be of a great interest to better understand the biological consequences of these differential selective constraints.

Conflict of interest

The authors declare that they have no conflict of interest.

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393

394 **Figure legends**

395 **Fig. 1:** Maximum likelihood tree of DELLA proteins (model JTT+F+R10). Tree was rooted using three
396 SCARECROW (SCR, belonging to another subfamily of GRAS transcription factor) sequences from
397 liverworts *Marchantia polymorpha* and are collapsed in the “outgroup” clade. DELLA2, Monocots
398 DELLA1, Eudicots DELLA1.1 and DELLA1.2 are indicated by coloured branches as follow: blue, green,
399 orange, red, respectively. The first outer circle indicates the main Angiosperm lineages as described below
400 the tree. The second outer circle represents the presence/absence and the position of DELLA (red right
401 pointing pentagram) and GRAS (dark blue rectangle) domains. Size and position of symbols is proportional
402 to their length and position in the protein. The third outer circle illustrates the gene structure, extrapolated
403 from the annotation file when available. Blue squares represent gene exons, their size and position are
404 proportional to their length and positions in the gene. Branch support was tested using the UFB2 method and

were masked from the tree for readability reason. Support values can be retrieved in the Supplementary Data S3 corresponding to the rooted tree shown here in Newick format.

Fig. 2: Logos of functional DELLA domains generated for each phylogenetic clade. For DELLA2, strongly affected sites are indicated by red arrows. Size of letter is proportional to the amino acid conservation at a given site. Blank in the logo represents highly gapped sites in the alignment. Theoretical functional models are written in black above logos and essential amino acids for the interaction with GID1, as defined by Murase et al. (2008), are indicated in bold.

Fig. 3: Boxplot representing pairwise dN/dS comparisons among the DELLA clades. All pairwise comparison between all DELLA sequences were calculated. For each clade (X-axis), comparison with other clades and itself (white waves) are shown separately. The dN/dS ratios were estimated using the Yang & Nielsen method (Yang and Nielsen 2000).

Fig. 4: Maximum likelihood tree of DELLA proteins (model JTT+ R5) in the Papilionoideae clade (A). Alignment of DELLA functional domains mapped on the phylogenetic tree. Blue background is proportional to identity level for each site. Functional DELLA subdomains are indicated by red rectangles (B).

Supplementary Data

Supplementary Data S1: Protein sequences used for phylogenetic analysis

Supplementary Data S2: Protein sequences discarded prior the phylogenetic analysis (less than 200 amino acids)

Supplementary Data S3: Rooted tree presented in Figure 1 in Newick format.

Supplementary Table S1: Summary table of DELLA sequences repartition. For each species, sequence ID is indicated for each DELLA clade. Absence of sequence for a given species/clade is indicated by empty cells. In addition, order, family, reference and abbreviation used for each species are indicated in the last four columns. For *Vitis vinifera*, the DELLA2 sequence is indicated but not retained in the analysis since it seems that this sequence is missassembly.

Supplementary Table S2: Results of the branch analysis for signature of selection acting on the different DELLA clades.

Supplementary Table S3: Amino acid frequency matrices for each DELLA copy. Numbers represent percentage of each amino acid at each position in the DELLA functional domain. First column indicates the consensus of DELLA domain.

436 Supplementary Figure 1: Alignment of DELLA domains in DELLA2 Monocots and *Medicago truncatula* as
437 reference. Blue-coloured names represent sequences with a predicted DELLA domain by Pfam. The DELLA
438 functional domains are indicated by black squares with the theoretical sequence indicated below. *Musa*
439 *acuminata*, *Siprodela polyrhiza* and *Zostera marina* belong to diverse lineages of Monocots outside the
440 Poaceae clade.

441

Tree scale: 1

First outer circle legend

- Basal Angiosperms
- Monocots
- Basal Eudicots
- Eudicots

DELLA $\S$ LxYxVxxxxMAxVAxxLExLEx $\S$

DELLA2

TVHYNPxxLxxWxxxM

DELLA1
Monocots

DELLA1.1
Eudicots

DELLA1.2
Eudicots

DELLA2

Tree scale: 0.1

10 20 30 40 50 60 70

DE LLAALGYKKVRSSDNMADVAQKLEQQLEVMVMTGNAQEDGEST I ISHLAS +DTVHY +DPSD IGLYSWVQTM