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Characterization of normal and "albino" phenotypes in *Erythrina crista-galli*

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We present here a characterization of *Erythrina crista-galli* L. (syn: *Erythrina lamifolia* Jacq.) seedlings, obtained from a plant from the Botanical Garden of Pisa University. This plant produces seeds that, during germination, have shown two different seedling phenotypes: normal (NT, 75%) and "albino" types (AT, 25%). Albino seedlings survive only 8–9 weeks and their growth is dramatically reduced when compared with wild type seedlings. Biochemical investigations have shown that albino seedlings completely lack chlorophyll and carotenoids and also soluble sugar levels are lower than in the normal type. We have also conducted sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) experiments and silver staining analysis on different protein extracts from shoots and leaves of both phenotypes, and demonstrated strong differences in protein patterns. The almost total absence of putative small and large RuBisCo bands in albino seedlings should be emphasized. We have also microspectrophotometrically determined the DNA content of this species, as it was lacking in the literature, by means of the Feulgen method. The recorded chromosome count of the species is confirmed to be $2n = 42$, a number characteristic of the genus *Erythrina*. No variation in Feulgen absorption and in chromosome number is detectable between NT and AT seedlings. To better characterize this species and the two phenotypes, the complete nucleotide sequences of the internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S rDNA have been also reported and compared with analogous sequences of some woody *Leguminosae* present in the European Molecular Biology Laboratory (EMBL) database. The results of this initial report lead us to conclude that albinism, in our system, has probably occurred as a result of natural mutation, and should be connected to genetic factors rather than to environmental conditions.

Keywords: albinism; DNA content; ITS sequences; protein pattern; RuBisCO

Introduction

The genus *Erythrina* (*Leguminosae*) is distributed in tropical and subtropical regions of the world and has been used in traditional medicine for the treatment of various diseases, especially microbial infections (Mitscher et al. 1987; Pillay et al. 2001). These plants are known to be a rich source of bioactive alkaloids (Ghosal et al. 1971; Furukawa et al. 1976; Barakat et al. 1977; Cordell 1981) and flavonoids, mainly isoflavones, pterocarpans, flavanones and isoflavanones (Chacha et al. 2005). Some of these flavonoids have been found to display a variety of biological properties, such as antimicrobial (Mitscher, Okwute, Gollapudi, Drake et al. 1988; Mitscher, Okwute, Gollapudi & Keshavarz-Shokri 1988; Chacha et al. 2005; Yenesew et al. 2005), anti-HIV (Mckee et al. 1997), antibacterial (Tanaka et al. 2002, 2004; Sato et al. 2002; Rukachaisirikul et al. 2007), anti-inflammatory (Njamen et al. 2003, 2004) and anti-plasmodial activities (Andayi et al. 2006 and references therein).

In this paper we present physiological, cytological and molecular data on the seedlings derived from an *Erythrina crista-galli* L. plant living in the Botanical Garden of Pisa University. It has previously been observed that this plant always produces seeds giving two different seedling phenotypes: normal type (NT) and albino (AT), 75% and 25% respectively.

Albinism is a consequence of partial or complete loss of chlorophyll pigments and of incomplete differentiation of chloroplast membranes, and is typical of higher plants (Kumari et al. 2009 and references therein). Studies have shown that in *Nicotiana tabacum* L. the chlorophyll absence in albino plants is due to some punctiform mutations or deletions in the plastidial genome (Avini et al. 1989; Fluhr & Cséplo 1986; Svab & Malinga 1986). The albino phenotype can also be due to mutation of many different nuclear loci as shown in *Fagopyrum esculentum* Moench. by Ohnishi (1982), in *Rhizophora mangle* L by Klekowski & Godfrey (1989) and in rye by Ballesteros et al. (2009).

This type of recessive mutation normally leads to plastidial ribosome disappearance (Bradbeer et al. 1979), although the real mechanism responsible for this phenomenon is still unclear (Feierabend & Berberich 1991).

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Several studies were conducted to analyze the gene expression pattern of albino plants in comparison to normal type. RuBisCO is the most abundant protein involved in photosynthetic processes driving CO_2 into the biosphere. It is also one of the largest enzymes in nature, with a molecular mass of 560 kDa, a holoenzyme composed by eight large and eight small subunits (Spreitzer & Salvucci 2002). In land plants and green algae, the chloroplast *rbcL* gene encodes the large subunit, whereas a family of *rbcS* nuclear genes encodes nearly identical small subunits.

Results have shown that RuBisCO is not expressed, or is expressed at very low levels, in albino plants (Zubko & Day 1998; Ankele et al. 2005), suggesting a relationship between the absence of this protein and albinism.

We present here data on germination, growth rate, sugar levels, and protein patterns of leaf, shoot, and root derived from the two seedling types (NT or AT); moreover to better characterize this species and its mutant, nuclear DNA content, chromosome number and rDNA sequences are reported.

Materials and methods

Plant material

Seeds of *Erythrina crista-galli* L. were obtained from a tree living in the Botanical Garden of Pisa University. A total of 200 seeds were soaked for 12 h in water and than sowed in Petri dishes on a bed of sand saturated with distilled water. When root emergence was visible, seedlings were transferred into peat-based growing medium in 8 cm diameter pots. All the biochemical analyses were carried out in triplicate in 7-week-old plantlets. From germinating seeds, collected in three subsequent years, it was possible to observe that growing plantlets were distributed in two groups: NT (75%) and AT (25%). As control we used 200 seeds obtained from a plant living in the Botanical Garden of Corrientes (Argentina). All the seedlings derived from the Argentinean plant showed the normal phenotype.

Phenotypic characterization of plantlets

Leaf length was measured from petiole to the top of the organ while leaf width was measured in the larger portion of the leaf. Shoot and root length were measured from the residual cotyledons to the shoot apex or to the root tip respectively. The weight of each single organ was also measured. All the measurements were conducted in triplicate for normal as well as for albino types. All the phenotypic characterizations were carried out in 7-week-old plantlets.

Analysis of carbohydrates

Samples (0.1–1 g FW) were rapidly frozen in liquid nitrogen and ground to a powder, then sugars extracted as described by Tobias et al. (1992) and assayed for glucose, fructose and sucrose content through coupled enzymatic assay methods (Guglielminetti et al. 1995). The efficiency of the method was tested by using known amounts of carbohydrates. Incubations of the samples and standards were carried out at 37°C for 30 min. The reaction mixtures (1 ml) were as follows. Glucose: 100 mM Tris-HCl, pH 7.6; 3 mM $MgCl_2$; 2 mM ATP; 0.6 mM NADP; 1 unit Glc6P dehydrogenase; the A_{340} was recorded. Fructose was assayed as described for glucose plus the addiction of 2 units of PGI; the increase in A_{340} was recorded. Sucrose was first broken down using 85 units of invertase (in 15 mM Na-acetate, pH 4.6) and the resulting glucose and fructose were assayed as described above.

Recovery experiments evaluated losses taking place during the extraction procedures. Two experiments were performed for each metabolite by adding known amounts of authentic standards to the sample prior to the extraction. The concentration of the standards added were similar to those estimated to be present in the tissues in preliminary experiments. The recovery ranged between 93 and 108%.

Analysis of pigments

Pigments were extracted by incubating tissues (50–100 mg) in 1.5 ml 80% acetone for 1 week at 4°C in darkness. The absorbance of extracts was measured spectrophotometrically at 470.0, 663.2, and 646.8 nm. These absorbance values were used for calculation of chlorophyll *a*, chlorophyll *b*, and total carotenoids contents by means of formulae suggested by Lichtenthaler & Wellburn (1983) and Wellburn (1994).

Protein extraction and separation

Seedling tissues were dissected and collected from 7-week-old plantlets and extracted in 50 mM Tris-HCl buffer (pH 7.6 containing 10 mM DTT and 10% glycerol). Protein quantification was performed according to Guglielminetti et al. (1997). Equal amounts of protein (2μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% polyacrylamide gels following by conventional silver nitrate staining.

Nuclear DNA determination and chromosome number

Roots (1 cm long) from 10 germinating seeds of *Erythrina crista-galli* (NT and AT) were collected and fixed in ethanol–acetic acid (3:1, v/v) overnight. The apical portions were then squashed in a drop of 45% acetic acid after treatment with a 5% aqueous solution of pectinase (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C, according to Venora et al. (2002). After removal of the cover slips by the dry ice method, the slides were air dried and then hydrolyzed in 5 N HCl at room temperature for 30 min, stained with Schiff's reagent, and washed according to the method of Kotseruba et al. (2000). Squashes of *Vicia bithynica* L. root tips were stained concurrently with the

other slides and used as an internal standard. Feulgen absorptions were measured using a Leitz MPV3 integrating microdensitometer (Leitz, Wetzlar, Federal Republic of Germany); absorption measured in *V. bithynica* preparations was used to convert relative Feulgen arbitrary units into picograms of DNA, by assuming a 4C DNA content of 18.30 pg for *V. bithynica* (Frediani et al. 1992). For each of five different root meristems of the two phenotypes, 50 nuclei were scored. For the internal standard (*Vicia bithynica*) 20 nuclei were scored for three different root meristems. For chromosome number determination, 1 cm long roots of *E. crista-galli* were treated with 8-hydroxyquinoline for 2 h, then fixed, squashed, and stained according to Bitonti et al. (1996). For each phenotype at least five metaphases for each of five seedlings were analyzed.

DNA extraction and sequencing

Nuclear DNA was extracted and purified from roots of germinating seeds of *Erythrina crista-galli* (NT and AT) using a DNeasyPlant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Each phenotype was sampled twice. The entire region including internal transcribed spacer regions ITS1, 5.8S and ITS2 rDNA was amplified by standard polymerase chain reaction (PCR) using the primers ITS5 and ITS4 (White et al. 1990). Amplifications were carried out using the following parameters: 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 2 min, 72°C for 2 min, and finally 72°C for 5 min. The PCR-amplified DNA fragments showed a single band when examined on agarose gel. The PCR products were sequenced directly for both strands by using the automated sequencer ABI Prism 310 (Applied Biosystems, Foster City, CA, USA). Internal transcribed spacer sequences were aligned by the Clustal 1.83 software with default values (Thompson et al. 1994) with analogous sequences of some woody *Leguminosae*, present in the European Molecular Biology Laboratory (EMBL) database: *Tamarindus indica* L. (accession number AB378735), *Dalbergia sissoo* Roxb. ex DC. (accession number AF189023), *Ceratonia siliqua* L. (accession number AJ245575, AJ245576) and *Robinia pseudoacacia* L. (accession number AF467495). Alignments were carried out as daughter processes of BioEdit (Hall 1999), which was also used for sequence editing and manipulation.

Statistical analyses

All computations were performed with R 2.13.2 (R Development Core Team 2011) and R package mgcv version 1.6-1.

Results

Figure 1 shows a representative sample of the two 7-week-old *Erythrina crista-galli* phenotypes. Phenotypic data characterizing the two samples are reported in Table 1. NT plants present several large and green leaves, a high shoot and a long root. Conversely, albino plants show some small white leaves, and also the length of shoots is strongly reduced in comparison to NT while roots do not show significant differences. The differences are more evident when comparing organ weights. NT leaves are about 10 times heavier than AT samples. Also roots and shoots are heavier in NT than in AT (3/4 times and 2 times, respectively).

Figure 2 illustrates data on pigment content. The levels of chlorophylls *a* and *b*, and carotenoids are reported for leaf or shoot samples from both AT and NT plants. It is evident that all the pigments are almost absent in leaves and shoots of albino plants, while high levels of chlorophylls *a* and *b* and a moderate level of carotenoids are detectable in NT leaves. Low levels of all three pigments are also present in the NT shoots. The chlorophyll differences are statistically significant, while differences in carotenoids do not show significance between NT and AT shoot and leaf.

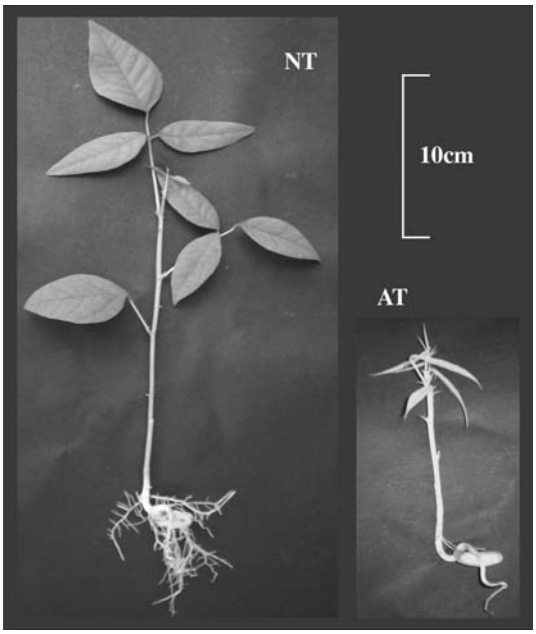

Figure 1. *Erythrina crista-galli* plants (7 weeks old).

Table 1. Phenotypic data of 7-week-old *Erythrina crista-galli* plants.

		NT(mean ± SD)	AT(mean ± SD)
Leaf length	(mm)	48.8 ± 19.9	19.8 ± 10.4*
Leaf width	(mm)	19.9 ± 11.3	2.0 ± 1.2*
Shoot length	(mm)	205.0 ± 7.1	105.0 ± 21.2*
Root length	(mm)	97.5 ± 24.7	57.5 ± 31.8
Leaf weight	(mg)	1046.0 ± 6.0	133.0 ± 53.0*
Shoot weight	(mg)	1009.0 ± 12.0	514.0 ± 78.0*
Root weight	(mg)	421.0 ± 53.0	150.0 ± 35.0*

*AT data are significantly different to NT data.

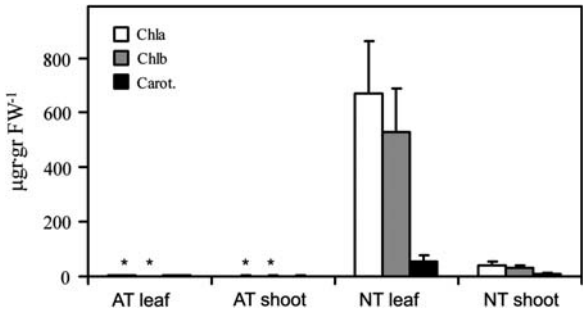

Figure 2. Pigment content in 7-week-old *Erythrina crista-galli* shoot and leaf. Chla = chlorophyll *a*; Chlb = chlorophyll *b*; Carot. = carotenoids; AT = albino type; NT = normal type. * indicates AT data are significantly different to NT data.

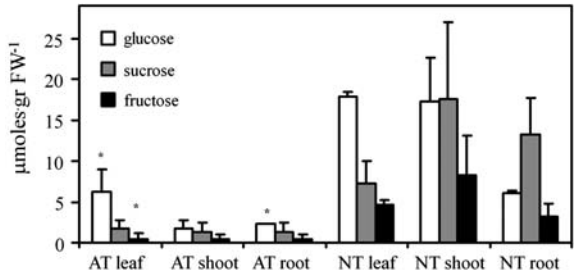

Figure 3. Soluble sugars content in 7-week-old *Erythrina crista-galli* shoot, root and leaf. AT = albino type; NT = normal type. * indicates AT data are significantly different to NT data.

Data on soluble sugar levels (glucose, fructose and sucrose) in the different *Erythrina crista-galli* organs after 7 weeks of germination are reported in Figure 3. In general sugar amounts are lower in the AT organs than in NT ones. Glucose is the most abundant sugar in all the albino samples while sucrose represents the most abundant sugar in all the NT samples with the exception of leaves, where glucose is higher.

Concerning protein separation after SDS-PAGE, AT and NT protein patterns obtained from leaf and shoot extracts after silver staining are shown in Figure 4. In shoots the large RuBisCO subunit (the band of about 55 kD) is strongly expressed in NT samples while only a weak band is present in AT samples at the same molecular weight both in AT1 and in AT2. In leaf extracts the intensity of the same band increases dramatically while no signal is detectable for the AT sample. In addition the small RuBisCO subunit (a band of about 12 kD) is evident exclusively in the NT leaves. It is also worth noting, in both organs, several bands of about 35 kD differentially expressed depending on the plant type.

As regards DNA analysis, the Feulgen absorptions, determined in prophase nuclei of root tips using *Vicia bithynica* preparations as an internal standard, are comparable within the two samples; and the nuclear DNA content is 7.98 ± 0.02 pg/4C nucleus. This determination is the first reported in the literature for this species. The 2*n* chromosome number of *E. crista-galli* was 42 in both the

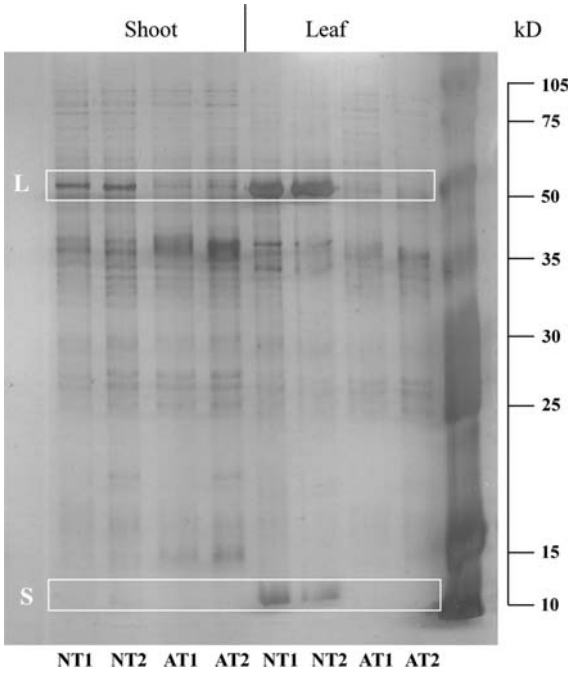

Figure 4. Protein patterns of AT and NT leaf and shoot extracts (12.5% SDS-PAGE and silver staining). NT = normal type; AT = albino type. All samples were loaded in duplicate (1 or 2). L = putative RuBisCO large sub-unit; S = putative RuBisCO small sub-unit.

phenotypes (Figure 5). This basic chromosome count of $n = 21$ is typical of the genus (Atchinson 1947). Both chromosome number and DNA content are higher than other reported data concerning some key woody *Leguminosae* (Table 2).

The nucleotide sequences of ITS1, 5.8S and ITS2 rDNA (Figure 6) are available in the EMBL database, with the accession number FN825780; no relationships between albinism and ITS sequences have been found since they are identical in NT and AT. The ITS1 region is 224 bp long with 50.4% GC content. The ITS2 region is 233 bp long and has 50.7% GC content. The 5.8S rDNA is 164 bp long with 51.8% of GC content. In Figure 6 the obtained sequences are compared to analogous sequences of some woody *Leguminosae*, present in the EMBL database (accession numbers reported in Table 2). A low homology is present if we compare ITS1 and ITS2 sequences; conversely we have an high level of homology when analyzing the 5.8S rDNA alignments: sequence identity was 90.24% for *Ceratonia siliqua*, 96.30% for *Robinia pseudoacacia*, 95.09% for *Dalbergia sissoo* and 94.44% for *Tamarindus indica*.

Discussion

Albinism in *Erythrina crista-galli*, as in all higher plants, can be determined by one or by a concomitance of two or more factors including genotype, environment, meiotic abnormalities, hormonal imbalance, nuclear–plastid genome incompatibility, deletion in plastid DNA, mutation

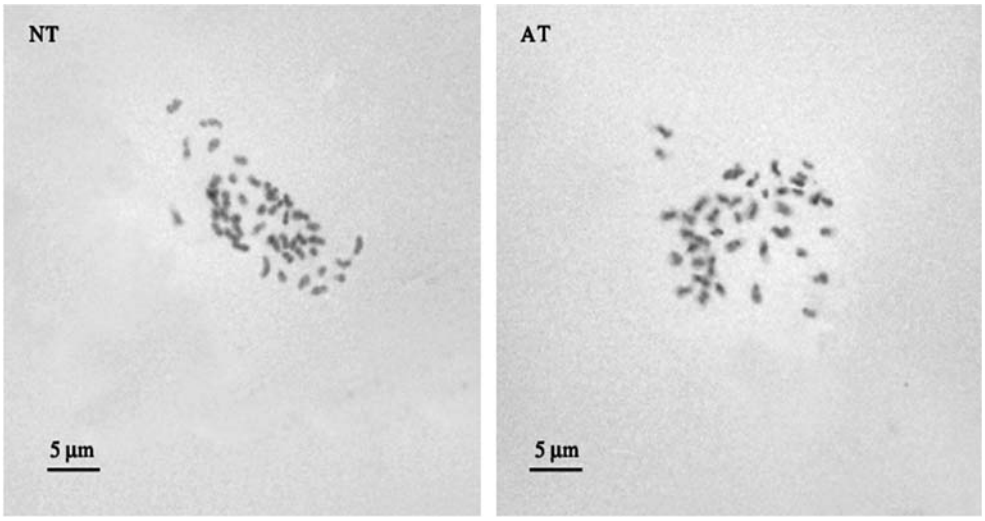

Figure 5. Metaphase plate from normal type (NT) and albino type (AT) obtained from cytogenetic analysis of *Erythrina crista-galli* root meristem.

Table 2. Chromosome number, mean nuclear DNA content and accession number of rDNA sequences in *E. crista-galli* and related species.

Species	Chromosome number ($2n$)	DNA amount (pg/4C nucleus)	Accession no.
Erythrina crista-galli L.	42	7.98 ± 0.02	FN 825780
Tamarindus indica L.	26[1]	3.35[1]	AB 378735[2]
Dalbergia sissoo Roxb. ex DC.	20[1]	2.82[1]	AF 189023[3]
Ceratonia siliqua L.	24[4]	2.48[5]	AJ 245575/76[6]
Robinia pseudoacacia L.	20[7]	2.60[7]	AF 467495[8]

[1]Ohri et al. 2004; [2]Sukrong et al. 2008; [3]Lavin et al. 2000; [4]Batlle & Tous 1997; [5]El Ferchichi Ourda et al. 2008; [6]Domenech-Sanchez et al. 1999; [7]Ceccarelli et al. 1998; [8]Hu et al. 2002

in genes responsible for chlorophyll biogenesis and/or functioning of photosynthetic apparatus (Kumari et al. 2009 and references therein). For these reasons, in this study we have compared *Erythrina crista-galli* AT lethal mutants with NT for several morphological and physiological traits and our results can be considered a first step in the analysis of the possible factors that can concur to albinism phenotype in this system.

One point of interest in the study of potential factors affecting albinism in plants is the control of ploidy level variations, which often depend on meiotic irregularities and abnormalities (Kim et al. 2003; Kumari et al. 2009 and references therein). To determine ploidy levels in our two systems we counted the chromosome number and estimated possible changes in nuclear DNA content by Feulgen absorption. This is a simple but accurate procedure that reflects real values in the nuclear DNA content, since the analyzed nuclei were chosen in the same post-synthetic conditions from tissues in the same developmental stages and all squashes were stained concurrently with *V. bithynica* root meristems, used as an internal standard. It is worth nothing that our determination of DNA content provides the first data regarding *E. crista-galli* and our values are in line with the determinations of Ohri et al. (2004) on *Erythrina* genus. No differences in chromosome number and in the mean Feulgen absorption between NT and AT type were detectable and these results are an indication that the albino phenotype, at least in our system, seems not to be related to difference in the chromosome number and/or in genome size due to variation of repetitive sequences that are cytophotometrically detectable. Obviously the method we have used does not allow recognition of small differences in DNA content due to changes and/or deletions in specific sequences of the nuclear genome.

Our studies focused as well on some morpho-physiological traits of albinism in plants. Phenotypic data are considerably different between NT and AT plants, the latter showing typical features of albinism, e.g. a strong reduction both in the growth level and in the organ weight, a reduced number of leaves and the typical white color. The reported lack of chlorophyll production (Borner & Sears 1986) is confirmed for our albino samples and also carotenoid level in albino tissues is very low. As a consequence the severe lack of pigment observed in *E. crista-galli* AT necessarily damages photosynthetic process and blocks plant growth causing plant death.

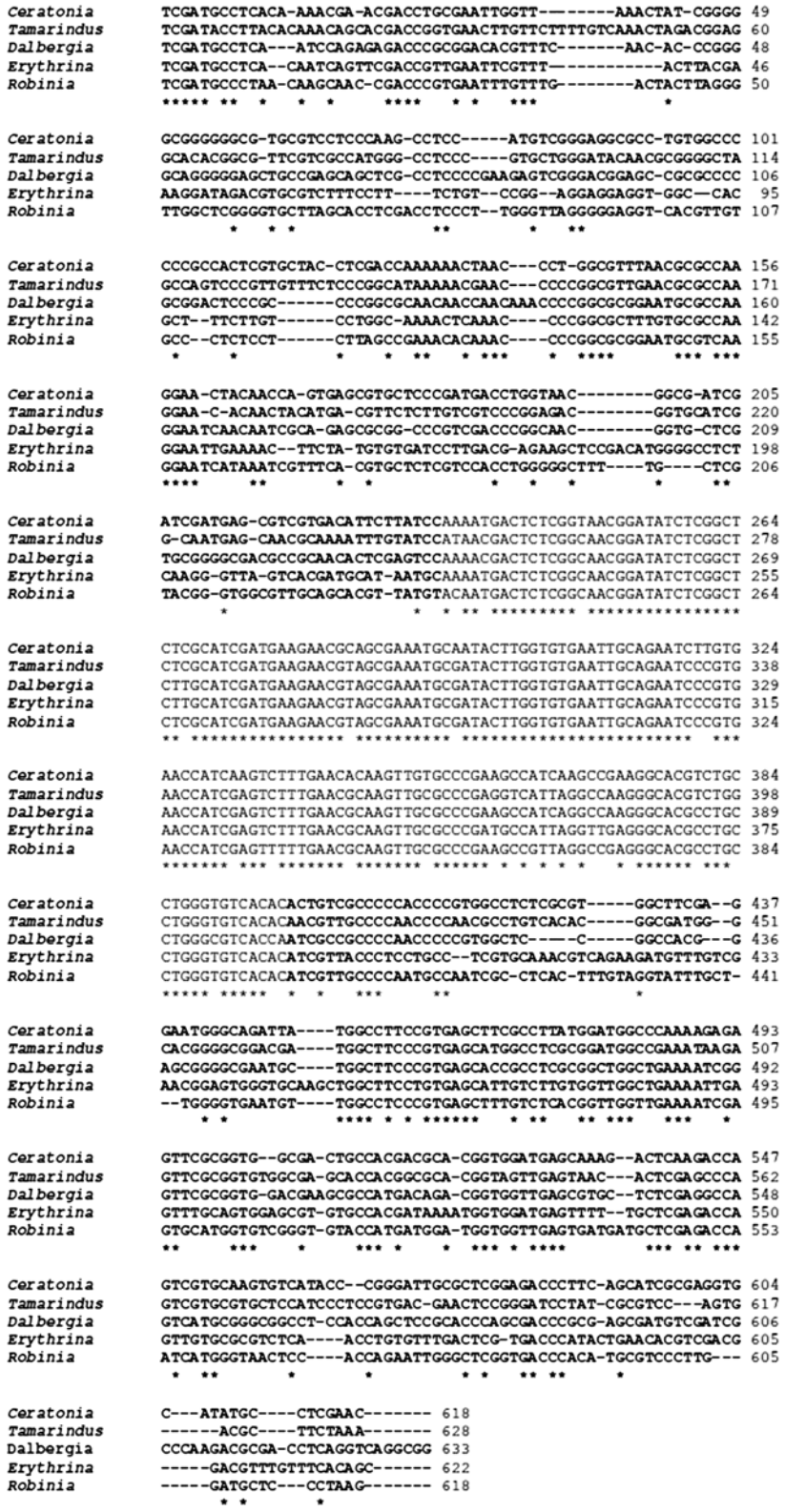

Figure 6. Nucleotide sequence comparison of the ITS 1, 5.8S and ITS 2 regions of the rDNA in *Erythrina crista-galli*, *Tamarindus indica*, *Dalbergia sissoo*, Ceratonia siliqua and *Robinia pseudoacacia*. In normal type nucleotide sequence of 5.8S rDNA; in bold type nucleotide sequence of ITS 1 and ITS2.

We have also analyzed RuBisCO levels in leaf and shoot tissues. Both large and small subunits of RuBisCO are almost absent in albino tissues, as reported by Zubko & Day (1998) for *Nicotiana tabacum*, some *Brassica* species and *Arabidopsis thaliana* L. (ecotype Columbia) and by Ankele et al. (2005) for cereals. It is to be under-

line the presence of a weak band corresponding to the large subunity of RuBisCO, coming from protein extracts belonging only to AT shoots and completely lacking in AT leaves. Taking into account that the two enzyme subunities of RuBisCO are encoded in two different genomes, nuclear and chloroplast genome expression may differently affect albinism in our system and this imbalance may vary depending on the plant organ. As a consequence of chlorophyll and RuBisCO absence, albino seedlings cannot grow autotrophically and are quickly subjected to sugar starvation that becomes lethal at 8–9 weeks. In contrast, in *Oryza sativa* it is possible to obtain a greenable albino mutant, which is able to survive temporarily as an albino until the 3-leaf-stage, in defense against the adverse environmental conditions (i.e. low temperatures); after that the mutant is able to begin photosynthetic metabolism, turning gradually green and completing its lifecycle (Xia et al. 2006).

In our system we have not observed albinism reversion phenomena; however, the very low level of sucrose (the normal soluble sugar transport form in plants) in AT plantlets could be explained by additional inefficient sugar translocation due to a general carbohydrate metabolism imbalance.

To better characterize *E. crista-galli* DNA, we have also determined the nucleotide sequences of a region of the ribosomal cistrons, i.e. the internal transcribed spacer regions (ITS1 and ITS2), separated by the 5.8S ribosomal RNA gene. ITS sequences have been used extensively to characterize a wide range of organisms, including fungi and plants, and have successfully been used to determine phylogenetic relationships among the taxa. The ITS regions of *E. crista-galli* present a very low homology with analogous regions of some woody *Leguminosae* and no relationship is detectable, as expected, between ITS sequences and the albino phenotype, since such sequences are identical in NT and AT samples.

The 5.8S rDNA sequence of *E. crista-galli*, if compared with the analogous regions of the above reported species, shows high level of sequence identity, ranging from 90.24% in *Ceratonia siliqua* to 96.30% in *Robinia pseudoacacia*, and confirming the idea that 5.8S rDNA, inside the rDNA sequences, is highly conserved.

In conclusion, albinism in our system has probably occurred as a result of natural mutation, and should be connected to genetic factors rather than to environmental conditions. The occurrence of AT (25%) versus NT (75%), taking place year by year, suggests that these two phenotypes are probably linked to a recessive trait governed by one gene with two alleles. Control of albinism by a single gene has been previously described in several taxa, including *Leguminosae* (Barwale & Widholm 1987; Neuffer et al. 1997; Shreekumari & Abraham 2005; Gettys & Wofford 2007). Further multidisciplinary studies are in progress to better characterize this system, aiming to clarify this highly genotype-dependent NT/AT ratio in *E. crista-galli*.

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