Arundo donax L.

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Arundo donax L., the giant reed—being a long-duration, low-cost, non-food energy crop able to grow in marginal lands—has emerged as a potential alternative to produce biomass for both energy production, with low carbon emissions, and industrial bioproducts.

Arundo donax L. reed genomic sequencing

1. Introduction

A. donax L. is a widespread species of unclear origin. This perennial grass grows spontaneously in temperate and tropical zones almost all over the world [1][2]. It can be found in ecosystems highly altered by anthropic activity and along riparian zones [3], where it often acts as an invasive weed reducing biodiversity [4], and brings an increased risk of wildfires and floods [5]. The roots can grow to 5 m in depth [1][6] and canes can reach 8–10 m in height and 3–4 cm in diameter (Figure 1). The leaves are flat, 5–8 cm wide and 30–100 cm long, inserted alternately in two ranks [1][7][8]. In southern Europe, new canes sprout continuously from rhizomes, starting in early March until August to November, when flowering takes place. Senescence follows in winter, with canes becoming yellow and generally losing leaves and inflorescences. Inflorescences are large plume-like panicles 30–100 cm long [7] that do not produce viable seeds [9][10][11][12][13].

Figure 1. Crop field of *A. donax L.* for energy purposes in the third year of cultivation.

Studies on *A. donax L.* sterility are often contradictory. In fact, this topic has yet to be clarified, since a drastic founder effect could explain this, rather than it being a consequence of defective chromosome pairing in aneuploid

A. donax and *A. micrantha Lam.* [14]. At first glance, in *A. donax,* male and female gametogenesis fails right after meiosis. Following the megaspores' mother cell formation at the tetrad stage, three chalazal megaspores degenerate, while one micropilar megaspore enlarges, develops a large nucleolus but no embryo sac, with the consequent proliferation of dysfunctional cells and the failure of ovule development. Pollen grains' cell walls usually collapse by autolysis, with the appearance of large numbers of vacuoles and variable numbers of nuclei and micronuclei. Despite this being the common result, the formation of a few viable pollen grains is reported with a frequency of 6.2% [14][15]. Meiosis occurs in less than 10% of microsporocytes, and no formation of exine occurs in the microsporangium [11]. A. donax sterility has been reported to be related to alterations in gametogenesis and fertilization and post-fertilization development [16]. *A. donax* sterility most likely has various causes that have led to its agamic propagation strategy. Reproduction is exclusively asexual and occurs through vegetative propagation [17][18] by fragmentation of rhizomes and cane fragments, which are dispersed by floods or by human activity [1][19].

The worldwide spread of *A. donax* is related to several domestic and agricultural purposes such as the making of walking-sticks, baskets, mats, fishing rods, fences, plant stakes and musical instruments' parts, especially the reeds for clarinets and saxophones [1][8][20][21]. *A. donax* is supposed to have spread from Asia, its native center, to America, passing through the Mediterranean area [15][22][23][24][25]. Other authors suggest that this plant originated in Mediterranean regions from native species [26]. At least four other species from the Arundo taxon are present in the Mediterranean area: *A. plinii L., A. collina Ten., A. mediterranea,* and *A. micrantha Lam*. [27][28]. Six lineages of *A. donax* are supposed to be distributed from Asia to the Mediterranean basin, with a putative area of origin in the Western and Southern edges of the Qinghai-Tibet Plateau [29]. The phylogenesis of *A. donax* is still debated, as the hypothesis that this species is polyploid or allopolyploid is shared by various authors based on its macroscopic traits, such as the great vegetative vigor and the absence of fertile seeds [12][13][30]. The literature data about the chromosome number of *A. donax L.* show some discrepancies, from an often-reported number of 108–110 chromosomes [12][30][31][32][33], to 84 chromosomes [13], or seed-producing cytotypes with 72 chromosomes, although this last result was published before the revised classification of the genus Arundo [28].

Large-scale cultivation of *A. donax* was established between the 1930s and the 1960s in Northern Italy to produce the textile fiber Rayon, but after the Second World War, it became unsustainable due to the competition from petroleum-derived products [1][34]. Recently, the economic interest in this species has risen again and a considerable number of publications have dealt with different topics about this interesting crop: bioenergy, agronomy, invasiveness, and its use for phytoremediation [35]. The high yield in dry matter per hectare and the low inputs required for cultivation make A. donax a promising energy crop [8][36][37]. Chips and pellets for direct combustion are a practical solid fuel obtainable from A. donax [38], while other possibilities are gasification [39], second-generation bioethanol [40][41], and biogas for co-generation. For the last-named use, various experiments in northern Italy have promoted *A. donax* as an acceptable substitute of *Zea mays L.* in anaerobic digestion plants. Other potential benefits include biofuels [42][43], biocompounds for plastic polymers [44], green building technologies [45] and leaf protein concentrate as a feed ingredient [46].

The capacity of *A. donax* to grow on marginal and abandoned lands makes this plant viable for cultivation on soil not suitable for traditional agriculture. Lands can be defined as marginal for different reasons, such as water scarcity, poor soil quality (e.g., high conductibility, low organic matter, etc.), and industrial pollution. On sandy loam soil (77% sand) with low organic matter content (1.2% organic matter) and low nutrients availability, the *A. donax* dry biomass yield was reported to be about 20 tha^{-1} [47]. This result was obtained with no irrigation, weeding or pest control. Taken together, these characteristics take this energy crop out of competition with food/feed cultures. Furthermore, *A. donax* has been classified as moderately salt tolerant with a 50% yield reduction at 11 dS m^{-1} salinity concentration [48]. Also, in this case, there is no competition with food-feed crops because, for these crops, the salinity concentrations determining 50% yield reduction are significantly lower, as in the case of corn (5.9 dS m^{-1}) and rice (3.6 dS m^{-1}) or similar as in the case of sugar cane (8–12 dS m^{-1}). For sugar beets, it is reported a higher salt tolerance (15 dS m^{-1}) but this culture requires a strong weeding control, irrigation and fertilization procedures.

Marginal lands are growing worldwide due to anthropic activities, in fact secondary salinization affects 20% of irrigated land worldwide [49], and in Europe, the soil contaminated with heavy metals represent 6.24% (137,000 km^2) of the total agricultural land [50]. This could mean an opportunity to cultivate energy crops environmental friendly such as A. donax in the near future.

2. Genetic Engineering

So far, to our knowledge, no transgenic *A. donax* plants with improved characteristics have been developed, possibly due to the limited regeneration of tissues and the absence of traits of interest well characterized at the molecular level. In any case, transient expression of GUS and GFP reporter genes is obtainable by an optimized particle bombardment protocol on Arundo donax callus cells. Important parameters to be taken into account include helium pressure, distance from stopping screen to target tissue, value of vacuum pressure, material and size of the microparticles, DNA concentration and number of bombardments. Higher efficiency in DNA transfer, resulting in 100–150 modification positive spots for explant, is achievable with cells bombarded twice at 1100 psi, with 9 cm target distance, 24 mm Hg vacuum pressure, 1 mm gold particle size, 1.5 μg DNA per bombardment, three days of pre-culture before the bombardment and six days of culture after bombardment. Bombardment with a GFP reporter gene resulted in higher expression than using GUS gene. The 35s promoter of CaMV can be used for the constructs, with hygromycin resistance to select modified cells [51]. Transformation of *A. donax* is also possible through protoplast manipulation. Starting from suspension culture, cell walls can be digested with a solution containing food-grade enzymes and 2–3 h incubation at 37 °C. The comparison among promoters highlighted that Ubi2 promoter from P. virgatum L. is a stronger promoter than CaMV 35S, with the second inducing low expression in A. donax. Trials with a different set-up of PEG-mediated transformation failed, while electroporation carried out at 130 V and 1000 μF resulted in a transformation efficiency of 3.3% ± 1.5% [52].

The study of the *A. donax* genome is still a demanding research topic aimed at collecting knowledge needed for *A. donax* genetic improvement, since the lack of a high-quality reference genome sequence. A hybrid approach combining Illumina and long-read sequencing technologies, i.e., Pac Bio or Nanopore, could be used as previously reported for de novo sequencing in other crops [53][54]. In particular, the high-quality reference genome of Oryza longistaminata has been obtained incorporating Illumina and PacBio sequencing data [53], while the de novo genome sequence assembly of trifoliate yam (Dioscorea dumetorum) was the result of Illumina and Oxford Nanopore technologies [54].

A parallel approach to identify putative target genes for *A. donax* genetic improvemt is the utilization of S. italica genome, the more related species that is actually sequenced. In fact, considering the lignin biosynthetic pathway genes, in particular PAL-like and CCoAMT-like genes, the high homology of four Mediterrean ecotypes of *A. donax* transcripts with *S. italica L.* [55] represent an important perspective for mining possible target sequences about this trait improvement by bioinformatics. A similar approach has been recently applied to isolate potential gene target to be used for genetic improvement of Miscantus × giganteus, a promising lignocellulosic biomass crop for biofuel production. Transcriptional analyses and phylogenetic and genome synteny analyses have allowed the identification of the major monolignol biosynthetic genes and the putative transcription factors regulating their expression [56] (Zeng et al. 2020).

Different reviews have been published on genetic improvement of energy crops, but for the energy crops suitable for genetic transformation, such as swichgrass, important results in the improvement of energy production have been obtained by gene silencing [57][58] and CRISPR/CAS 9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9) [59] techniques. However, the data reported for A. donax are based on the results of agronomic studies with the aim of optimization of biomass quality.

Currently, sequence databases and information on organs diversity and possible targets for improvement can be found in RNA-seq Illumina transcriptomics studies. Gene Ontology Analysis of metabolic differences among bud, culm, leaf and root tissues highlighted that the most variety can be found in the leaves, most notably for light, osmotic, salt and metal stress response, and for primary and secondary metabolites production [60]. About 40–45% of transcripts showed homologies with known sequences and functional annotations of *Oryza sativa L., Triticum aestivum L.* and mostly with *S. bicolor L*. and *Z. mays L.*, most importantly for gene categories related to flowering time, plant height and structure, carbohydrates composition and vernalization response. CCoAMT-like genes deserve particular attention for their possible role in obtaining mutants with decreased content of lignin in culms [60]. The response of *A. donax* to low oxygen stress analyzed by a metabolomic approach [61] provided numerous insights required to target functional genes by transcriptomics.

About the well-known *A. donax* tolerance to low soil quality, RNA-seq provided insights of the available defenses from adverse soil conditions. Considering the excess of Ni and Cu, doses of 25–100 mg/L activated the expression of a metal-uptake YSL-like gene and a macrophage protein which was NRAMP-like [62]. Adjustments in phytochelatin synthases expression could represent a reliable strategy to increase *A. donax* uptake of metalloid contaminants with the purpose of phytoremediation. With an RNA-seq methodology, three putative genes, AdPCS1-3 have been identified in *A. donax*. The expression of these three genes in response to $CdSO_4$ stress was tissue specific, with AdPCS1 the most up-regulated compared with control. However, the production of *Arabidopsis thaliana L.* transgenic lines overexpressing these genes resulted in deleterious effects on growth, with necrotic effect, while the same strategy applied to yeast resulted in Cd-tolerant lines [63]. The responses to salt stress and salt tolerance are other important traits investigated by RNA-seq with improvement purposes. Different ecotypes exhibited a possible positive correlation of salt exposure with the expression of stress-induced transcription factors DREB2A-like and WRKY53-like, activation of detoxification processes and abscisic acid increase. Moreover, a fast response to salt stress, with overexpression of ion transporters and K+/Na+ homeostasis-related genes, such as SOS1-like, NHX1-like or KHT1-like, represents an effort to reduce the ionic stress, but was detrimental to the growth performance [64][65]. A RNASeq analysis [66] conducted under long-term salt stress allowed the identification of differentially expressed genes with a dose-dependent response. The analysis was performed on a total 38,559 DEGs (differentially expressed genes) and among them, 2086 were up-regulated and 1766 were down-regulated.

In particular, it is reported the analysis of clusters related to salt sensory and signaling, hormone regulation, transcription factors, Reactive Oxygen Species (ROS) scavenging, osmolyte biosynthesis and biomass production. Several unigenes identified have the potential to be used to improve productivity and stress tolerance in *A. donax*. In particular, the silencing of the GTL1 gene (a homolog of Setaria italica trihelix transcription factor) acting as a negative regulator of water use efficiency could be a good target for NBT (new breeding techniques).

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