Chapter 13

ROLE OF ALTERNATIVE SPLICING IN CHICKPEA (*CICER ARIETINUM* L.) DROUGHT TOLERANCE MECHANISM, REVEALED VIA TRANSCRIPTOME ANALYSIS

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DOI https://doi.org/10.30525/978-9934-26-389-7-13

INTRODUCTION

Within the agricultural ecosystem, grain legumes serve as an integral component. Representing one of the most ecologically pivotal and diverse botanical families, legumes are indispensable for crop rotations and intercropping strategies due to their proficiency in nitrogen assimilation via a symbiotic association with rhizobia. Specifically, *Cicer arietinum*, or chickpea, stands as the third most prominent grain legume, surpassed only by *Pisum sativum* L. (field pea) and *Phaseolus vulgaris* L. (common bean). Chickpea grains has a composition of 17–24 % protein and 41–51 % carbohydrates, and they are replete with essential minerals, vitamins, dietary fiber, folate, β-carotene, antioxidants, and key micronutrients such as phosphorus, calcium, magnesium, iron, and zinc, along with linoleic and oleic unsaturated fatty acids[1].

Drought is the most prevalent abiotic stressor affecting plants, impairing their water equilibrium and constraining their growth and maturation. Intrinsically, chickpea genotypes possess mechanisms to combat drought stress through strategies such as escape, avoidance, and tolerance. A variety of breeding techniques, encompassing hybridization, mutation, marker-assisted breeding, genome sequencing, and omics methodologies, hold promise for enhancing chickpea germplasm resilience to drought stress. Among the morphological attributes, root depth and biomass are particularly instrumental in mitigating terminal drought stress in chickpea. Marker-assisted selection, a form of genomics-assisted breeding, can substantially

[1] Singh R., Singh C., Ambika et al. Exploring chickpea germplasm diversity for broadening the genetic base utilizing genomic resourses. URL: https://pubmed.ncbi.nlm.nih.gov/36035111/

augment the precision and efficiency of crop breeding. Emphasizing the significance of such breeding technologies, including marker-assisted breeding, omics techniques, and insights from plant physiology, underscores the potential for their incorporation in future breeding initiatives, aiming to develop drought-resilient chickpea cultivars[2].

While there is substantial knowledge regarding the physiological responses of chickpea genotypes that are tolerant or sensitive to cold, heat, and drought, this understanding has not been fully covered by genetic and genomic evidence. Advances in genomics and transcriptomics have expanded our comprehension of the genes and their regulatory networks associated with cold, drought, and heat stress in chickpea. Nonetheless, this understanding remains partial, as it has yet to coalesce into clearly delineated pathways that provide tolerance or susceptibility to these predominant abiotic stresses in chickpea[3].

Chickpea genome is 738 Mb and a total of 28,269 annotated genes, located in eight homologous chromosome pairs (2n = 16)[4, 5]. Transcriptomics studies suggest involvement the up-regulation of genes implicated in the photo-phosphorylation process (such as transferases, oxygen lyases, and oxidoreductases), hormone pathways (encompassing brassinosteroids, abscisic acid, and gibberellin responses), solute transportation, nutrient assimilation, and cell wall characteristics (including cellulose synthases, hemicellulose synthases, polygalacturonases, and pectate lyases) in drought tolerance[6].

One of the most promising and interesting meachanisms of drought tolerance within plants involves alternative splicing (AS)[7]. So investigation of AS involvement into drought tolerance within chickpea is actual and important task for unraveling chickpea drought tolerance mechanisms and its

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deeper understanding, which provide valuable information for further improvement of chickpea genotypes drought tolerance via marker-assisted selection.

1. Materials and methods

For transcriptome analysis four transcriptomes of two chickpea genotypes (Negussu et al., 2023): Desi PI598080 – susceptible for drought and Kabuli Flip07 318C – drought tolerant, non-treated and treated for imitation of drought stress were used.

ERR11526167 was transcriptome of Desi PI598080 in control conditions and ERR11526168 in stress conditions, ERR11526172 was transcriptome of Kabuli Flip07 318C in control conditions and ERR11526176 in stress conditions.

De novo assembly was performed by program Trinity[8]. For *de novo* transcripts assembly evaluation was performed differential expression analysis on gene level. Differential expression analysis assembled transcriptomes clustering was performed by program CD-HIT[9], map reads was performed by program Salmon[10], differential expression analysis was performed by library DESeq2[11] and NOISeq[12, 13]. For visualization, standard functions of R programming language[14] were used.

For AS differential expression analysis, SUPPA software was used[15, 16].

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2. Results

Four total *de novo* transcriptomes was assembled. Kabuli Flip07 318C in control conditions (Kabuli/Control transcriptome) counted 37494 transcripts total, in stress conditions (Kabuli/Stress transcriptome) 38797 transcripts total, Desi PI598080 in control conditions (Desi/Control transcriptome) 38985 transcripts total and in stress conditions (Desi/Stress transcriptome) 37500 total.

Differential expression analysis was performed with Kabuli/Control-Kabuli/Stress transcriptomes combination, Desi/Control-Desi/Stress transcriptomes combination, Kabuli/Control-Desi/Control transcriptomes combination, Kabuli/Stress-Desi/Stress combination and combination of all four.

Transcriptomes combination MA plots, a scatter plots used to visualize the differential expression of genes between two experimental conditions, are shown on figure 1 (A, B, C, D), where 'M' denotes the log2 ratio of expression levels between two experimental conditions or samples, while 'A' signifies the average log2 expression level across the conditions. Each point on the plot corresponds to a single gene. Genes that are differentially expressed appear as points significantly above or below the horizontal line at M=0, indicating up-regulation or down-regulation, respectively. Genes with higher average expression are situated towards the right of the plot, while genes with lower expression are found towards the left. Distribution of points varied and depended both on genotype, and experimental conditions. Kabuli/Control-Kabuli/Stress showed lesser up-regulation and down-regulation as it shown denser points near M=0 line, whereas Desi/Control-Desi/Stress showed more drastic changes, with more genes both up-regulated and down-regulated, as it was illustrated in study Negussu et al. (2023). Desi/Control-Kabuli/Control showed differential expression pattern closer to Desi/Control-Desi/Stress than Kabuli/Control-Kabuli/Stress, the main difference being lesser up-and down-regulation of higher expressed genes, whereas Desi/Stress-Kabuli/Stress showed more similar with Desi/Control-Desi/Stress differential expression pattern with bigger down-regulation, which suggests that genotype influence is also a major factor in differential expression pattern.

The results highlight a subset of genes showing significant differential expression, depending both on genotype and on experimental condition, which are of particular interest for further biological interpretation and validation.

MA plot, combining all four experimental variables (genotype and experimental conditions) is shown on figure 2. MA plot dots pattern contained numerous statistically significant differentially expressed genes, which suggested that AS was involved.

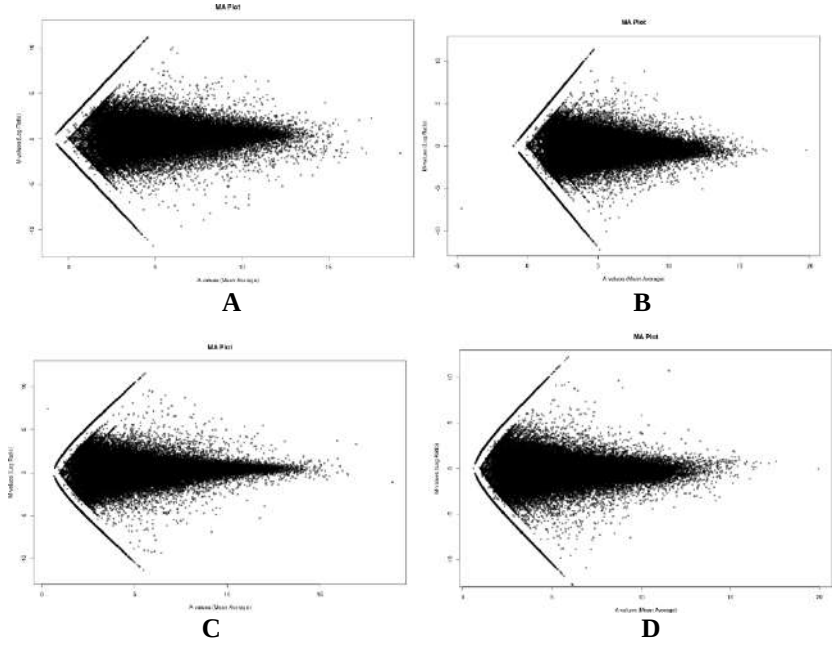

Fig. 1. Transcriptomes combination MA plots:

A – Desi/Control-Desi/Stress; B – Kabuli/Control-Kabuli/Stress;
C – Kabuli/Control-Desi/Control; D – Kabuli/Stress-Desi/Stress

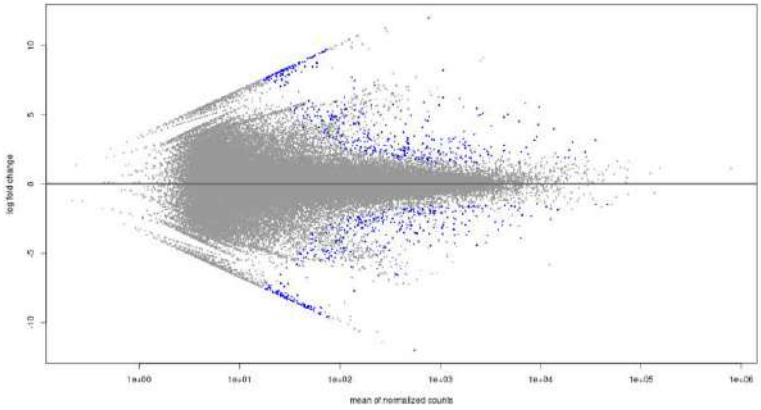

Fig. 2. MA Plot of Gene Expression Data. Blue dots show statistically significant differential expression

For each type of AS events, PSI (Percent Spliced In) was analyzed. he PSI provides a robust parameter to quantify AS events, facilitating a nuanced understanding of their role in post-transcriptional regulation. In our research, such AS events were analyzed:

1. A3: Alternative 3' Splice Site

Alternative 3' splice site (A3) events entail the selection of different 3' splice sites within the same intron, leading to the generation of transcript variants with alterations in their 3' regions. We calculated PSI values for A3 events to assess the differential usage of 3’ splice sites, providing insights into how these events may influence protein function and localization by altering their C-terminal regions.

2. A5: Alternative 5' Splice Site

Alternative 5' splice site (A5) events involve the utilization of distinct 5' splice sites within the same intron, resulting in transcript variants with variations in their 5' regions. By analyzing PSI values for A5 events, we aimed to elucidate the extent of 5’ splice site usage, shedding light on potential impacts on protein N-terminal regions, signal peptide processing, and subcellular targeting.

3. AF: Alternative First Exon

In alternative first exon (AF) events, different first exons are spliced to a common set of downstream exons, creating transcript variants with unique 5' ends. We calculated PSI values for AF events to explore the differential usage of first exons, providing insights into their potential roles in modulating transcriptional regulation, mRNA stability, and translation initiation.

4. AL: Alternative Last Exon

Alternative last exon (AL) events involve the use of different last exons, leading to transcript variants with distinct 3’ ends. By analyzing PSI values for AL events, we sought to uncover the extent of last exon usage, aiming to understand its implications for mRNA stability, polyadenylation, and the generation of protein isoforms with varied C-terminal domains.

5. MX: Mutually Exclusive Exons

Mutually exclusive exons (MX) events are characterized by the inclusion of one exon while excluding another from the mature transcript, resulting in the generation of protein isoforms with distinct functional domains. We employed PSI values to quantify the inclusion levels of exons involved in MX events, aiming to decipher their role in enhancing proteomic diversity and functional adaptability.

6. RI: Retained Intron

Retained intron (RI) events occur when an intron that is normally spliced out is retained in the mature transcript. Through the calculation of PSI values for RI events, we aimed to investigate the prevalence of intron retention and

its implications for mRNA stability, translation efficiency, and the potential generation of novel protein isoforms.

Comparative data of PSI values is shown in Table 1

Table 1

PSI values for combinations

Combination of Genotype/Condition	Event type	Count	Mean	50 %	75 %	Max
Desi/Contol (DC)	A3	3539.0	0.515161	0.528337	0.852561	0.989292
	A5	1765.0	0.470517	0.454324	0.790402	0.986095
	AF	488.0	0.50406	0.508574	0.768324	0.98752
	AL	215.0	0.501635	0.497098	0.70671	0.973369
	MX	214.0	0.493831	0.512421	0.590593	0.968901
	RI	1815.0	0.549862	0.572885	0.796383	0.986736
Desi/Stress (DS)	A3	2910.0	0.522800	0.547936	0.84366	0.989499
	A5	1443.0	0.459879	0.424459	0.762513	0.985559
	AF	397.0	0.523116	0.508984	0.778382	0.990097
	AL	210.0	0.470896	0.476052	0.684376	0.962483
	MX	209.0	0.504808	0.501845	0.603444	0.932781
	RI	1485.0	0.557127	0.583141	0.799969	0.989116
Kabuli/Control (KC)	A3	3359.0	0.525702	0.556092	0.845822	0.989197
	A5	1758.0	0.461441	0.433069	0.773575	0.986783
	AF	453.0	0.504968	0.500000	0.797005	0.988362
	AL	236.0	0.530452	0.512946	0.736677	0.989765
	MX	211.0	0.509322	0.510104	0.607373	0.938743
	RI	1693.0	0.554853	0.584061	0.791038	0.979439
Kabuli/Stress (KS)	A3	3538.0	0.536142	0.580460	0.854654	0.989271
	A5	1775.0	0.461409	0.446469	0.778564	0.985771
	AF	405.0	0.516577	0.510640	0.778920	0.987187
	AL	213.0	0.512093	0.493666	0.719169	0.973536
	MX	223.0	0.507506	0.500000	0.616303	0.936644
	RI	1937.0	0.562738	0.587054	0.794394	0.988220

Histograms of comparative analysis of PSI values for all events are shown on Figure 3.

In control conditions, both Desi PI598080 and Kabuli Flip07 showed overall similar total count for AS events. For Desi PI598080 genotype the drop of total count of AS events was typical, whereas for Kabuli Flip07 slight increase in AS events was typical.

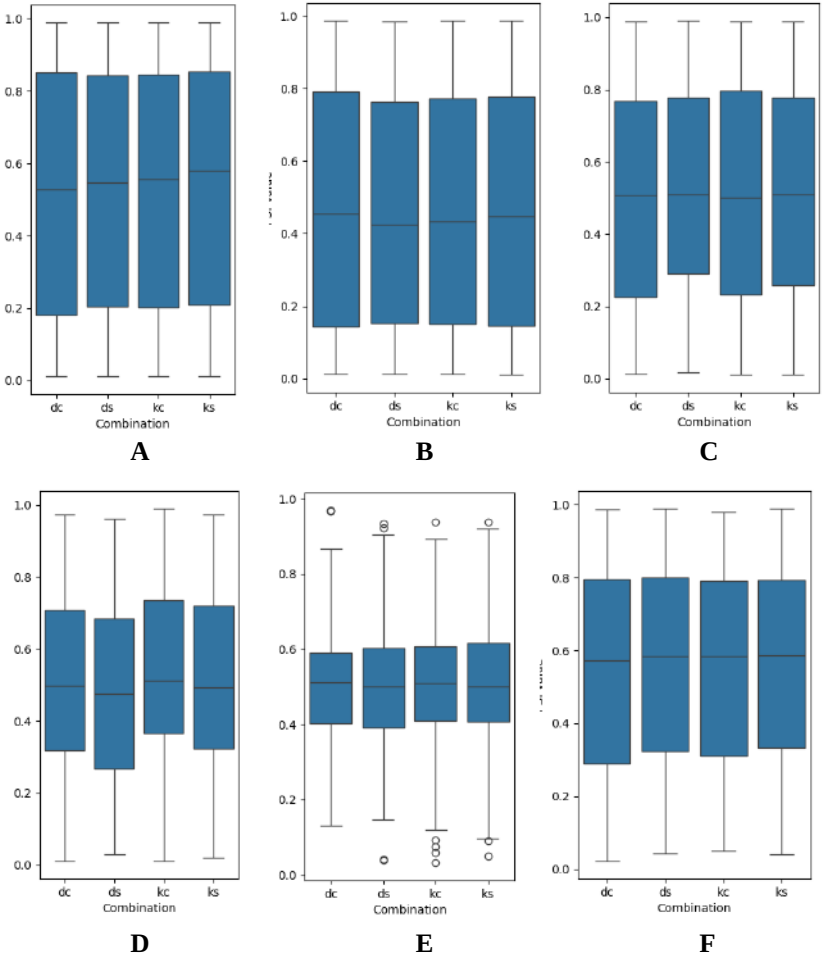

Fig. 3. Comparative analysis of PSI values:

A – A3 events; B – A5 events; C – AF events; D – AL events; E – MX events; F – RI events

3. Discussion

De novo assembled transcripts analysis of differential expression showed results in concordance with research Negussu et al. (2023) – sensitive genotype (Desi PI598080) showed aggravated levels of genes/pathways

modulated by drought, which can be attributed to both the fact that these plants experience a higher stress because of dis-adaptations of sensitive genotype on anatomical level, as well as the fact that tolerant genotype (Kabuli Flip07) is already have optimal transcriptome profile for abiotic stress, which leads to lesser number of both up-and down-regulated genes. Such anatomical level adaptations for drought stress in chickpea are deep, penetrating root system, reduced leaf size, smaller xylem vessels diameter. Generally, Desi chickpeas tend to have a more compact plant growth habit with smaller leaves and shorter stature, adapting well to diverse growing conditions. Kabuli chickpeas generally exhibit a more upright growth habit, with larger leaves and a taller stature, requiring more optimal growing conditions, whereas Desi PI598080 and Kabuli Flip07 showed reverse reaction to abiotic stress, which can suggest that in these genotypes adaptation was on physiological level rather than on purely anatomical one.

In the sensitive genotype, we noted a widespread alteration in gene expression, encompassing both lower and higher expressed genes. This extensive transcriptional reprogramming suggests a broad and potentially intense response to drought stress, impacting various cellular processes and pathways, as was discussed in [6]. The up-regulation of genes, irrespective of their baseline expression levels, indicates an active mobilization of the plant's resources to mitigate the effects of stress. Conversely, the down-regulation of highly expressed genes could reflect a reallocation of energy and resources away from typical growth and developmental processes, aiming to bolster the plant's defensive mechanisms.

Contrastingly, the tolerant genotype exhibited a more nuanced response, predominantly affecting lower expressed genes. This pattern suggests a targeted and efficient stress response, potentially pointing to specific pathways or responses known to confer resilience under drought conditions. By sparing the highly expressed genes, which are often involved in fundamental cellular functions and growth, the tolerant genotype appears to maintain its developmental processes more effectively under stress, contributing to its enhanced tolerance. This efficiency in response not only aids in immediate survival but may also contribute to sustained productivity under prolonged drought conditions.

While analyzing differential AS, in our research Desi PI598080 genotype showed overall drop in total count of AS events, whereas Kabuli Flip07 showed increase in some. Desi genotype, which is more susceptible to drought stress, leading to a reduction in its ability to maintain or increase alternative splicing under stress conditions. Alternative splicing is a crucial mechanism for increasing transcriptomic and proteomic diversity, allowing plants to adapt to various environmental stresses. The reduction in AS events could mean that

the Desi genotype has a limited capacity to adjust its splicing machinery in response to drought, potentially making it less resilient. In contrast, the Kabuli Flip07 genotype exhibits a slight increase in AS events under drought stress. This indicates a more robust response to drought, potentially contributing to its tolerance. The increase in AS events could facilitate the production of stress-responsive proteins or modify existing proteins' functions to help the plant cope with the adverse conditions.

The nuanced response of the Desi PI598080 and Kabuli Flip07 genotypes to drought stress, as evidenced by their distinct AS profiles, underscores the complexity of post-transcriptional regulation in plant stress adaptation. For the Desi PI598080 genotype, the observed reduction in AS events aligns with its known susceptibility to drought, suggesting a potential link between AS plasticity and drought resilience. This drop in AS events may result in a less diverse mRNA and protein repertoire, limiting the plant's ability to fine-tune gene expression and protein function in response to water scarcity.

The decrease in AS events in Desi PI598080 could specifically impact the production of functionally diverse protein isoforms, which are often crucial for signaling, osmotic adjustment, and cellular homeostasis under stress conditions. This could, in turn, compromise the cellular integrity and adaptive responses of the Desi PI598080 plants, rendering them more vulnerable to drought-induced damage.

On the other hand, the Kabuli Flip07 genotype's ability to maintain or even increase AS events under drought stress reflects a potential molecular strategy for enhancing stress tolerance. The increase in AS events might be driving the generation of novel transcript variants and protein isoforms, equipping the Kabuli plants with a broader molecular toolkit to combat drought stress. This enhanced splicing flexibility could play a pivotal role in modulating gene expression patterns, adjusting metabolic pathways, and activating stress response networks, contributing to the overall resilience of Kabuli Flip07 to water-limited conditions.

Interestingly, the differential AS observed across genotypes may also have implications for the long-term adaptation and evolutionary trajectories of these chickpea cultivars. The Kabuli genotype's splicing plasticity under drought might be a result of selective pressures and adaptive evolution, leading to the establishment of a more robust splicing machinery capable of navigating the challenges of water scarcity. Conversely, the Desi genotype's limited splicing flexibility under stress conditions may reflect a trade-off or a different adaptive strategy, potentially shaped by its unique evolutionary history and ecological niche.

Traditionally, Kabuli chickpeas are recognized for their upright growth habit, larger leaves, and taller stature, traits often correlated with a need for

optimal growing conditions. However, in the context of abiotic stress, particularly drought, the responses of these two Kabuli genotypes have been anything but typical.

Desi PI598080, despite its usual robustness and tolerance to suboptimal conditions, exhibited a pronounced susceptibility to drought stress. This was manifested not just in its physiological responses, but also at the molecular level, as evidenced by a marked reduction in alternative splicing events. Such a decline in post-transcriptional regulation complexity could potentially translate to a diminished capacity for rapid molecular adaptation, leaving the Desi genotype vulnerable in the face of environmental challenges.

Contrastingly, Kabuli Flip07 demonstrated an unexpected resilience, increasing its alternative splicing events under drought conditions. This molecular adaptability, often less attributed to Kabuli varieties due to their preference for optimal conditions, suggests an unanticipated layer of complexity in the drought response mechanisms of this genotype. The ability to enhance transcriptomic and proteomic diversity under stress could be a key determinant of Kabuli Flip07's resilience, aiding in the maintenance of cellular homeostasis and the activation of adaptive pathways.

Furthermore, the nuanced responses of Desi PI598080 and Kabuli Flip07 to abiotic stress underscore the importance of considering both phenotypic and molecular traits in crop improvement programs. While phenotypic characteristics provide a visible and direct measure of adaptation, the underlying molecular responses are crucial in painting a comprehensive picture of the plant's resilience mechanisms.

In conclusion, our findings highlight the integral role of alternative splicing in mediating plant responses to drought stress, with distinct AS patterns correlating with varying levels of drought resilience across chickpea genotypes. This research not only sheds light on the molecular underpinnings of drought response in chickpea but also provides a valuable framework for future investigations aimed at unraveling the complex web of post-transcriptional regulation in plant stress adaptation. Ultimately, deciphering the functional consequences of AS in stress conditions will be crucial for developing innovative strategies to enhance crop resilience in the face of changing environmental conditions.

CONCLUSIONS

The adaptations of chickpea plants to drought conditions are multifaceted, involving alterations in root system architecture, leaf anatomy, xylem vessel characteristics, and cellular osmotic adjustment. These adaptations collectively confer drought tolerance, ensuring survival, and productivity under water-limited conditions. Elucidating these mechanisms is paramount

for advancing breeding efforts aimed at improving drought tolerance in leguminous crops, contributing to food security and sustainable agricultural practices.

The observed gene expression patterns highlight the complexity of the plant's response to drought stress and underscore the genetic basis of drought tolerance in chickpea. These findings have profound implications for breeding programs, pointing towards the potential for developing chickpea varieties that combine the resilient stress response of the tolerant genotype with desirable agronomic traits. Further research, including functional validation of key differentially expressed genes and pathway analyses, is imperative to unravel the precise molecular mechanisms at play and to facilitate the translation of these findings into tangible agricultural benefits.

Alternative splicing is a versatile regulatory mechanism that allows for the production of multiple mRNA variants from a single gene, resulting in an expanded proteome and enhanced functional diversity. In plants, alternative splicing plays a crucial role in development, signal transduction, and stress responses, including drought tolerance. Alternative splicing introduces transcriptome plasticity, generating protein variants that contribute to cellular functions under drought conditions.

The identification of drought-responsive alternative splicing events can provide molecular markers for marker-assisted selection in breeding programs aimed at enhancing drought tolerance.

Alternative splicing emerges as a vital regulatory mechanism in chickpea's response to drought stress, contributing to the plant's resilience and adaptability under water-limited conditions. Understanding the specific alternative splicing events and their functional implications is paramount for developing chickpea varieties with enhanced drought tolerance, ensuring sustainable production and food security.

SUMMARY

Chickpea is a crucial crop for global food security. However, drought presents a major abiotic stressor, adversely affecting chickpea's growth and productivity. The plant has evolved a repertoire of strategies including escape, avoidance, and tolerance to mitigate the impacts of drought, with root architecture and biomass playing a crucial role in this resilience. Alternative splicing emerges as a key regulatory mechanism, contributing to transcriptome plasticity and functional diversity, which are essential for the plant's adaptability under water-limited conditions. The identification of drought-responsive alternative splicing events paves the way for the development of molecular markers, facilitating marker-assisted selection in breeding programs aimed at enhancing drought tolerance.

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