Conditional up-regulation of cytokinin increases growth and development of sugarcane during water deficits

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Abstract Globally 70% of sugarcane is grown under rain-fed conditions, and water deficits are the largest production constraints in sugarcane. Hormones are the key chemical messengers that control various plant processes, including growth, development and abiotic stress responses. Water stress elicits local and systemic changes in hormones abscisic acid, ethylene, cytokinin, auxin, gibberellins, jasmonic acid and brassinosteroids. Currently, considerable research effort is directed to understand the role of hormones, particularly cytokinin, abscisic acid and ethylene in plant adaptation to abiotic stresses, including water stress. It is hypothesized that water deficit decreases cytokinin production to reduce growth and water consumption by accelerated leaf senescence and shoot growth. To test that hypothesis conditional modification of cytokinin level in sugarcane (Saccharum spp. hybrid) under water deficit was studied via two different strategies: exogenous supply of a synthetic cytokinin, 6-benzylaminopurine, and up-regulation of cytokinin biosynthesis (transgenic approach) in response to water deficit. Up-regulation of cytokinin levels during water stress was achieved by driving the cytokinin biosynthesis regulatory enzyme, 2-isopentenyltransferase (IPT), by water stress or abscisic acid-responsive promoters. Results showed that exogenous supply of cytokinins and stress-induced expression of IPT significantly improved sugarcane growth and survival during water deficits. These results indicate the potential of hormonal regulation of water stress responses in sugarcane.

Key words Sugarcane, water stress, cytokinin, isopentenyltransferase

INTRODUCTION

Nearly 70% of the world’s sugarcane is grown in rain-fed conditions, and, over the year-long crop cycle, commercial crops often experience moderate to severe water deficits for varying duration. Water limitation is predicted to increase globally with climate change, causing significant economic loss. For instance, in the Australian sugar industry, loss of revenue due to water stress is about AUD260 million per year (Inman-Bamber 2007). Despite the economic importance, there is no dedicated breeding program for improving water-use efficiency and drought tolerance, primarily due to the lack of reliable selection traits and cost-effective screening methodologies.

Large, multi-location field-based sugarcane germplasm screening experiments showed potentially exploitable genetic variation for growth and yield in responses to water stress (Hemaprabha et al. 2006; Basnayake et al. 2012). However, very little is known about the mechanistic understanding of this growth and yield variation in sugarcane. Substantial inhibition of shoot growth, early senescence and reduction in photosynthesis has been reported in water stress-affected sugarcane (Basnayake et al. 2012; Lakshmanan and Robinson 2014). The plant hormones abscisic acid, ethylene and cytokinins are considered as key regulators of water-stress responses where cytokinin plays an important role in controlling leaf senescence and photosynthetic capacity (Ha et al. 2012).

Based on the variation in senescence observed in water-stressed plants grown in the field, we hypothesized that a water stress-induced decline in cytokinin content and the consequent reduction of chlorophyll may, at least in part, account for the decline in photosynthesis and growth inhibition. We tested this hypothesis using two approaches: (i) exogenous application of cytokinin to a commercial sugarcane cultivar; and (ii) transgenic lines of the same sugarcane cultivar capable of producing cytokinin in response to water stress.
MATERIALS AND METHODS

We used an Australian sugarcane cultivar Q208\(^{\text{f}}\) and achieved endogenous cytokinin modification by two strategies:

1. Exogenous supply of N6-benzyladenine (BA; 100 µM), a synthetic cytokinin, via root application.
2. Conditional up-regulation of endogenous CK levels by expressing a transgene encoding a cytokinin biosynthesis regulatory enzyme, 2-isopentenytransferase (IPT), driven by senescence-associated (SAG12) or abscisic acid-responsive (RAB17) promoters (Fig 1).

![Diagram of transgene constructs showing the locations of (A) conditional expression cassette; the promoter, isopentenytransferase coding sequence (IPT CDS) and terminator and (B) cassette for selection; the promoter, neomycin phosphotransferase II (nptII) gene and terminator.](attachment:image)

**Transformation**

Transgenic constructs were introduced into sugarcane callus via microprojectile bombardment (Bower and Birch 1992). Biolistic transformation was performed using 1 µm gold particles coated with either (A) whole circular plasmid containing the expression cassette (P\(_{\text{SAG12}}\)::IPT and P\(_{\text{RAB17}}\)::IPT), or (B) cassette for selection (P\(_{\text{Ubi}}\)::nptII, supplied by Sugar Research Australia) referred to as PDNA (plasmid DNA) in this paper (Joyce et al. 2014). Each expression cassette (P\(_{\text{SAG12}}\)::IPT and P\(_{\text{RAB17}}\)::IPT) was co-bombed with P\(_{\text{Ubi}}\)::NPTII that allows for antibiotic selection of transformed cells using Geneticin (A.G. Scientific, Inc. USA). Co-transformed embryogenic calli were selected on MS medium (Murashige and Skoog 1962) with 3 mg/L 2,4-D and 50 mg/L Geneticin (Sigma, USA) in the dark for 4 weeks, and then transferred to a 16-hour photoperiod for plant regeneration (medium with no growth regulators but with the same Geneticin concentration). Shoots were regenerated after 8-12 days. Tissue culture controls (TC) consisted of regenerated plants which had undergone a similar treatment as their transgenic counterparts, except for the omission of the vector in the transformation process and regeneration on culture medium containing no antibiotics (Joyce et al. 2014). From each callus one plant was selected and labeled as an independent line. We generated 113 independent transgenic lines. Plantlets with roots were transferred to potting trays with peat moss and sand (60:40 v/v) and grown in a PC2 glasshouse under natural light at 30-35°C. After 30 days of acclimation, healthy plantlets of similar size were transferred to 0.7 L pots (Garden City Plastics, Australia) in peat moss and sand (60:40) for water-stress experiments.

**Measurements**

Chlorophyll content index (CCI) and final biomass were measured in sugarcane plants grown in a glasshouse with soil moisture maintained at around 50% field capacity via daily irrigation. CCI was measured once a week in the middle portion of the youngest fully expanded leaves using CCM-200 plus chlorophyll meter (Opti-Sciences, Inc., USA). Total biomass (dry weight of shoot + root) was collected at the end of the experiment. In this research, a wild-type plant (WT, Q208\(^{\text{f}}\)), a WT supplied with cytokinin (WT+CK), three independent P\(_{\text{RAB17}}\)::IPT transgenic lines; RAB19, RAB20 and RAB25 and a P\(_{\text{SAG12}}\)::IPT transgenic line, SAG32, were studied under water stress conditions for 50 days where each pot consisted of...
approximately 2500 (± 50) g of peat and sand (60:40 v/v) and covered with 2 cm polyethylene beads (Qenos Pty Ltd, Australia) to minimize soil evaporation.

RESULTS

Chlorophyll content index (CCI) reduction in wild-type plants was more severe than that of cytokinin up-regulated and cytokinin supplied plants by day 36 (Fig. 2A). By the end of the experiment, CCI was nearly 40% higher in RAB25 and SAG32 plants than wild-type (Fig. 2A). Water stress caused a considerable reduction in plant height and total biomass in all plants after 50 days of treatment. However, there was considerable variation in stalk height among transgenic lines. RAB25 plants were significantly (P<0.05) taller (~12%) than wild-type plants, while RAB19 plants were significantly (P<0.05) shorter (~30%) than wild-type plants under water stress treatment (Fig. 2B). Total biomass was reduced in all six tested lines after 50 days under water stress conditions, but RAB25, SAG32, WT+CK and RAB20 had significantly (P<0.05) higher biomass than wild-type plants (50%, 40%, 31% and 20%, respectively) in the water-stress treatment (Fig. 2C).

**DISCUSSION**

Our results show that cytokinin plays a significant role in the maintenance of chlorophyll content in sugarcane. Plants receiving cytokinin exogenously or transgenic lines with up-regulated cytokinin production had improved biomass production under water deficit. The negative impact of water deficit on chlorophyll content was reduced by exogenous supply or transgenic up-regulation of cytokinin. Higher chlorophyll levels and possibly other cytokinin effects on maintaining the integrity of photosynthetic machinery (Qin et al. 2011) might have improved photosynthesis in RAB25 and SAG32 transgenic plants and plants that received exogenous cytokinin. This was reflected by the improved shoot growth and

Fig. 2. (A) Chlorophyll content index, (B) stalk height and (C) total biomass of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under water stress conditions. CCI data were collected once a week. The values are averages of six replications ±SE. Different letters at each panel (day 36, 43, 50 of experiment) indicate significant differences among lines at P<0.05.
biomass in cytokinin-supplied and transgenic plants under stress. Together these results provide evidence that cytokinin plays a role in water stress responses in sugarcane, and that upregulation of cytokinin during stress periods will reduce the negative impact of stress on growth and development.

ACKNOWLEDGEMENTS

We thank Dr Jaya Basnayake for assistance with the water-stressed experimental strategy, Dr Chuong Nguyen Ngo for gene expression studies and Jessica Vogt, Stéphane Guillou, Dr Richard Brackin, Dr Henrique Junqueira Franco and Leonard Leo for data collection.

REFERENCES


La régulation positive conditionnelle de cytokinine augmente la croissance et le développement chez la canne à sucre durant des déficits hydriques

Résumé. Sur le plan mondial, 70% de la plantation de canne à sucre est réalisée sous les conditions pluviales, et les déficits hydriques constituent la contrainte principale à la production cannière. Les hormones sont des messagers chimiques clés contrôlant plusieurs processus chez les plantes comme la croissance, le développement et les réponses aux stress abiotiques. Le stress hydrique provoque des changements hormonaux au niveau local et systémique chez l’acide abscissique, l’éthylène, la cytokinine, l’auxine, la gibbérelline, l’acide jasmonique, et les brassinostéroïdes. Actuellement, des nombreux travaux de recherche sont consacrés pour comprendre le rôle des hormones, en particulier la cytokinine, l’acide abscissique et l’éthylène, dans l’adaptation des végétaux aux stress abiotiques, y compris le stress hydrique. On pense que le déficit hydrique diminue la production de cytokinine afin de réduire la croissance et la consommation d’eau, causée par la senescence accélérée des feuilles et la croissance des pousses. Afin de vérifier cet hypothèse, on a étudié la modification conditionnelle du niveau de cytokinine dans la canne à sucre (Saccharum spp. hybride) sous stress hydrique, avec deux méthodes différentes; l’approvisionnement exogène de cytokinine synthétique, la 6-benzylaminopurine, et la régulation positive de la biosynthèse de cytokinine (une approche transgénique) en réponse au déficit hydrique. La régulation positive du niveau de cytokinine durant le stress hydrique a été réalisée à l’aide d’une enzyme de biosynthèse de la cytokinine, 2-isopentényltransférase (IPT), provoqué par le stress hydrique ou à l’aide de promoteurs sensibles à l’acide abscissique. Les résultats démontrent une amélioration considérable de la croissance et la survie chez la canne à sucre pendant le déficit hydrique grâce à l’approvisionnement exogène du cytokinine synthétique et l’expression induite d’IPT par le stress. Ces résultats indiquent le potentiel de la régulation hormonale au stress hydrique chez la canne à sucre.

Mots-clés: Canne à sucre, stress hydrique, cytokinine, isopentényltransférase

Condicional regulación de citoquina aumenta el crecimiento y desarrollo de la caña de azúcar durante el déficit de agua

Resumen. A nivel mundial el 70% de la caña de azúcar se cultiva en condiciones de secano, y el déficit de agua son los mayores obstáculos para la producción de caña de azúcar. Las hormonas son los mensajeros químicos que controlan diversos procesos de la planta, incluyendo el crecimiento, el desarrollo y diferentes respuestas al estrés abiótico. El estrés hídrico provoca cambios locales y sistémicos en las hormonas como etileno, citoquina, auxina, giberelinas, ácido abscisico y jasmonico y brasoenoesteroïdes. En la actualidad, un considerable esfuerzo de investigación está dirigido a comprender el papel de las hormonas, particularmente citoquininas,
ácido abscísico y etileno en la adaptación de las plantas al estrés abiótico, incluyendo el estrés hídrico. Se plantea la hipótesis de que el déficit de agua disminuye la producción de citoquinina para reducir el crecimiento y el consumo de agua en la senescencia foliar acelerado y el crecimiento de los brotes. Para probar esta hipótesis modificación condicional del nivel de citoquininas en la caña de azúcar (Saccharum spp. híbrido) se estudió bajo estrés hídrico a través de dos estrategias diferentes: suministro exógeno de una citoquinina sintética, 6-benzylaminopurin, y sobre regulación de la biosíntesis de citoquinina (enfoque transgénico) en respuesta al déficit de agua. Sobre regulación de los niveles de citoquinina durante el estrés hídrico fue alcanzado por la conducción de la biosíntesis de enzima citoquinina reguladora, 2-isopenteniltransferasa (IPT), por el estrés hídrico o promotores sensibles al ácido abscísico. Los resultados mostraron que la expresión inducida por el estrés suministro exógeno de citoquininas y de IPT mejoró significativamente el crecimiento de la caña de azúcar y la supervivencia durante los déficit de agua. Estos resultados indican el potencial de la regulación hormonal de las respuestas de estrés hídrico en la caña de azúcar.

**Palabras clave:** Caña de azúcar, estrés hídrico, citoquinina, isopenteniltransferasa