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IMPACT DES CONDITIONS DE CULTURE *IN VITRO* ET DU SACCHAROSE EXOGÈNE SUR LA RÉGULATION DE L'EXPRESSION GÉNIQUE ET L'ACCUMULATION DES PROTÉINES CHEZ LES PLANTULES DE TOMATE (*SOLANUM LYCOPERSICUM*)

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Résumé

La micropropagation est une technique efficace pour multiplier rapidement *in vitro* plusieurs espèces de végétaux. Cependant, les conditions de culture *in vitro* sont très différentes des conditions naturelles ce qui entraîne, lors du transfert *ex vitro*, la mortalité de plusieurs plantules. On sait que les conditions de culture *in vitro* et le saccharose exogène ont un effet majeur sur la photosynthèse et le succès lors de l'acclimatation. Dans cette thèse, nous avons étudié la réponse métabolique des plantules de tomate (*Solanum lycopersicum*) aux conditions de culture *in vitro* et à la présence de saccharose dans le milieu de culture. Dans un premier temps, nous avons observé que l'environnement *in vitro*, comparativement aux conditions *ex vitro*, entraîne la surexpression de gènes impliqués dans la photosynthèse et l'assimilation de l'azote, la sousexpression des gènes liés à la force des puits et l'induction d'une réponse de stress chez les plantules de tomates. Dans un deuxième temps, nous avons observé que le retrait du saccharose lors du transfert *ex vitro* stimule à court terme la biosynthèse de l'éthylène et initie une cascade signalétique causant une modification des propriétés de la paroi et une stimulation des gènes de défense. Dans un dernier temps, l'étude de l'impact à long terme du saccharose exogène sur le protéome des plantules à montrer un rôle central de ce facteur dans la régulation de la prolifération cellulaire, la photosynthèse et le mécanisme de défense contre les espèces réactives de l'oxygène. Globalement, la perturbation du métabolisme des espèces réactives de l'oxygène (ROS), de la paroi cellulaire, du système de défense, et du développement et de la division des cellules sont des réponses prédominantes chez les plantules en culture *in vitro* et lors de l'acclimatation. Ces données ont permis de mieux expliquer le phénotype des plantules *in vitro* et leur impact sur l'acclimatation devra inévitablement être mesuré dans le futur.

Résumé (version longue)

La micropropagation est une technique efficace pour multiplier rapidement plusieurs espèces fruitières, légumières et ornementales. Bien que la culture *in vitro* possède plusieurs atouts, la culture en milieu confiné a des conséquences néfastes sur la croissance et le développement des plantules. En effet, au cours du processus de micropropagation, les plantules vont modifier leur biochimie, leur physiologie et leur morphologie en réponse à l'environnement *in vitro* (Pospisilova *et al.*, 1999). Notamment, les conditions de culture *in vitro* se caractérisent par une concentration élevée en saccharose et en azote, une faible luminosité, une humidité relative élevée et une présence de CO_2 en quantité restreinte (Nguyen *et al.*, 2001). Malgré une phase d'acclimatation, lors du transfert *ex vitro*, le changement draconien de conditions environnementales occasionne un stress important qui réduit le taux de survie des plantules. Plusieurs études ont démontré un effet marqué des conditions de culture et du saccharose sur la photosynthèse et l'acclimatation (Desjardins, 1995; Fuentes *et al.*, 2005; Kubota *et al.*, 2001). Cependant, il existe peu de données pouvant expliquer les implications métaboliques globales des conditions *in vitro* et du saccharose exogène sur les plantules.

Pour s'attaquer à cette problématique, trois hypothèses ont été émises: i) l'expression génique des plantules en culture *in vitro* est très différente de celle de plantules ayant subi une acclimatation *ex vitro* et la croissance en conditions photomixotrophes stimule l'expression de gènes liés au métabolisme carboné et azoté, et à la réponse au stress et inhibe ceux impliqués dans la photosynthèse; ii) le transfert des plantules des conditions *in vitro* vers le milieu *ex vitro* produit une cascade signalétique engendrée par le retrait du saccharose et stimule, à court terme, les gènes liés aux signaux de transduction et à la régulation du développement; iii) la présence de saccharose exogène dans le milieu Murashige et Skoog (qui contient une forte teneur en N) interagit avec le métabolisme azoté et stimule la synthèse protéique et l'assimilation de l'azote. Pour réaliser ce projet de recherche, la tomate (*Solanum lycopersicum*) a été utilisée comme espèce modèle.

Pour tester la première hypothèse, nous avons observé l'impact de l'environnement *in vitro* sur l'expression des gènes grâce à une biopuce à ADN personnalisée de 122 gènes

et au PCR en temps réel. Ainsi, nous avons procédé à l'hybridation de l'ARNm de tissus foliaires de plantules cultivées en conditions *in vitro* et *ex vitro*. Comme prévu, l'expression génique des explants en culture *in vitro* s'est avérée très différente de celles des plantules *ex vitro*. L'environnement *in vitro* a entraîné la surexpression de gènes impliqués dans la photosynthèse et dans l'assimilation de l'azote, la sous-expression des gènes liés à la force des puits et l'induction d'une réponse de stress chez les plantules de tomates. En somme, ces résultats suggèrent que la présence de saccharose exogène est un facteur environnemental clé qui modifie l'équilibre oxydatif cellulaire et cause la modulation des gènes impliqués dans la réponse au stress et la physiologie anormale des plantules *in vitro*.

Nous avons par la suite évalué l'effet à court terme du retrait du saccharose du milieu de culture sur l'expression des gènes afin de mieux caractériser son impact lors du transfert *ex vitro*. Ainsi, des plantules ont été mises en culture pendant 20 jours *in vitro* en présence de saccharose et transférées en présence (3%) ou en absence de saccharose exogène pour une période de 24 h. À l'aide de la biopuce à ADN de la tomate, TOM2, nous avons mesuré le profil d'expression de 11 800 unigènes. L'utilisation du logiciel MapMan et une étude plus détaillée des données nous ont permis d'effectuer un classement ontologique des 251 gènes régulés à un niveau significatif. Étonnamment, après 24 h de carence en saccharose, les données de la biopuce à ADN ont démontré que le transcriptome réagit rapidement aux nouvelles conditions sans toutefois initier le catabolisme des réserves, la stimulation de la photosynthèse et sans causer l'arrêt de la croissance cellulaire. En revanche, les résultats obtenus suggèrent que le retrait du saccharose provoque une réorganisation structurelle et favorise la synthèse des nucléotides nécessaire à la synthèse pariétale tout en modifiant la membrane cellulaire. Ces résultats nous laissent croire que le saccharose inhibe la biosynthèse de l'éthylène et que son retrait du milieu de culture initie une cascade signalétique qui provoque une modification des propriétés de la paroi et stimule des gènes de défense.

Puisqu'aucune étude n'avait encore investigué la réponse à long terme et la variation de l'abondance en protéines de plantules mises en présence de saccharose, nous avons étudié l'impact du saccharose exogène sur l'abondance des protéines de plantules de

tomate par électrophorèse bidimensionnelle. Dix-sept protéines présentant une accumulation différentielle ont été identifiées par spectrométrie de masse. Comme prévu, l'ajout de saccharose dans le milieu MS a stimulé la synthèse protéique des plantules. Nous avons observé une augmentation de l'abondance des protéines impliquées dans l'élongation, le repliement et la dégradation des protéines, la synthèse de nucléotide et le transport mitochondrial chez les plantules mises en présence de saccharose. À l'inverse, l'abondance des protéines impliquées dans la chaîne de transport d'électron photosynthétique et la fixation du carbone, et une peroxydase ont diminué en présence de saccharose. Les paramètres photosynthétiques mesurés concordent avec la modification de l'accumulation des protéines observées. Une superoxide dismutase Cu/Zn et une glutathione peroxydase exprimée dans le chloroplaste ont affiché une diminution et une augmentation de leur abondance, respectivement. Ces résultats mettent en évidence le rôle primordial que joue le saccharose exogène dans la régulation de la prolifération cellulaire, la photosynthèse et le mécanisme de défense contre les espèces réactives de l'oxygène ROS.

Globalement, ces résultats montrent que la perturbation du métabolisme des ROS, de la paroi cellulaire et du développement et de la division des cellules sont des réponses prédominantes chez les plantules en culture *in vitro*. Certes, ces résultats permettent d'expliquer le phénotype particulier des cultures *in vitro*. En outre, les résultats de cette étude suggèrent que l'usage d'une étape d'acclimatation *in vitro* qui viserait à accroître les compétences photosynthétiques, à augmenter la force des puits et à restreindre le stress oxydatif lors du transfert *ex vitro* par la diminution de la concentration en saccharose exogène pourrait être bénéfique pour la survie des plantules.

Avant-Propos

Cette thèse est présentée sous forme d'articles scientifiques devant être publiés dans des revues scientifiques. Ma contribution pour chacun des articles est détaillée, ci-après :

Chapitre 2

Gene expression analysis reveals *in vitro* culture-induced regulation of primary metabolism and stress-related genes in tomato (*Solanum lycopersicum*) plantlets

J'ai réalisé l'ensemble des travaux qui ont permis l'écriture de cet article. J'ai effectué la préparation de la biopuce à ADN en collaboration avec Mme Andreja O. Preradov, ancienne étudiante à l'Institut des nutraceutiques et des aliments fonctionnels (INAF). La biopuce à ADN a été produite en collaboration avec Dr. Marc-André Sirard, chercheur à l'INAF. J'ai rédigé l'article sous la supervision de mon directeur de recherche Dr. Yves Desjardins. L'article sera soumis en avril, 2010 à la revue « Journal of Plant Physiology ».

Chapitre 3

Expression profiling of *in vitro* cultured tomato *(Solanum lycopersicum)* plantlets under sugar deprivation reveals important perturbation of ethylene signaling, cell wall metabolism and defense responses

J'ai réalisé la totalité des travaux qui sont présentés dans cet article. Les biopuces à ADN TOM2 ont été fournies par le « Center for Gene Expression Profiling» du Boyce Thompson Institute. J'ai rédigé l'article sous la supervision de mon directeur de recherche Dr. Yves Desjardins.

Chapitre 4

Proteomic approach toward a better understanding of the impact of exogenous sucrose in *in vitro* tomato (*Solanum Lycopersicum*) plantlets

J'ai réalisé l'ensemble des travaux qui ont permis l'écriture de cet article. L'analyse des protéines par spectrométrie de masse a été effectuée au « Centre d'innovation Génome

Québec et Université McGill ». J'ai rédigé l'article sous la supervision de mon directeur de recherche Dr. Yves Desjardins.

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Finalement, mais non le moindre, je dois souligner le support incontesté de mes parents et ma famille au cours de mes longues études. Merci à ma conjointe, Céline, pour son soutien indéfectible et ses encouragements dans les moments les plus difficiles.

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Chapitre 1 : Introduction

1.1 La micropropagation

À l'échelle mondiale, la culture *in vitro* présente des revenus de 15 milliards $US et la production de près d'un milliard de plantes annuellement. L'intérêt et la popularité de la micropropagation sont inhérents aux conditions de culture *in vitro* qui permettent d'exploiter efficacement la totipotence des cellules végétales, c'est-à-dire leur aptitude à se dédifférencier et former de nouveaux organes et éventuellement une nouvelle plante. La micropropagation est un outil efficace pour la multiplication d'espèces qui sont parfois difficiles ou impossibles à propager par les méthodes conventionnelles. En agriculture, la propagation *in vitro* est une technique qui s'avère efficace pour assurer la multiplication rapide de plusieurs espèces fruitières, légumières et ornementales. C'est aussi un outil essentiel à la régénération des plantes transformées génétiquement. L'objectif principal de la micropropagation est de produire une grande quantité de plantules génétiquement et physiologiquement identiques, au développement normal, exempte de pathogène, et ce, avec une bonne reprise *ex vitro*.

La culture *in vitro* (ou micropropagation) est une technique qui permet la propagation de cellules ou de tissues végétaux en milieu confiné. Les étapes clés de la micropropagation consiste en la sélection d'un explant, son implantation dans un milieu de culture, sa propagation par des cycles successifs de division et de repiquage et finalement, son transfert *ex vitro*, c'est-à-dire à l'extérieur du contenant. Malgré les nombreux avantages de la micropropagation, la culture en milieu confiné a des conséquences majeures sur la croissance et le développement des plantules. Durant leur séjour en culture *in vitro*, les plantules modifient leur métabolisme, leur physiologie et leur morphologie en réponse à leur nouvel environnement (Pospisilova *et al.*, 1999). Il est généralement reconnu que les changements induits par les conditions de culture *in vitro* sont la cause principale de leur dégénérescence *ex vitro*. Lors du transfert des plantules en conditions *ex vitro*, le changement drastique de conditions environnementales nécessite une adaptation majeure et occasionne un stress important qui affecte le taux de survie des individus.

1.1.1 Les conditions de cultures

La micropropagation des plantules en conditions *in vitro* nécessite l'utilisation d'un milieu de culture qui satisfait aux besoins en éléments minéraux majeurs et mineurs

essentiels à la croissance des plantes, et de manière facultative, des acides aminés, des vitamines, des phytohormones et des sucres. Le milieu de culture le plus couramment employé est sans contredit le milieu Murashige et Skoog (Murashige & Skoog, 1962). D'abord développé pour la culture du tabac (*Nicotiana tabacum)*, il convient à la micropropagation de plusieurs espèces. Ce milieu se distingue par sa forte concentration en azote, 60 mM, ce qui est environ 80 fois plus élevé que la concentration observée dans un sol typique. Plus précisément, ce milieu contient 40 mM de nitrate et 20 mM d'ammonium et provoque une croissance soutenue des plantes. La quantité de phosphore et potassium y est aussi élevée. Le milieu MS contient de plus des composés organiques comme la thiamine, l'inositol, la niacine et la pyridoxine. La culture peut-être réalisée sur milieu liquide ou solide, en ajoutant un agent solidifiant comme l'agar.

Au-delà du milieu de culture, la propagation en milieu confiné *in vitro* est réalisée dans des chambres de croissance et par conséquent, impose des conditions très différentes de celles trouvé naturellement. En effet, la culture *in vitro* se distingue par des conditions de faible luminosité, une présence de CO_2 en quantité restreinte, des échanges gazeux limités, une humidité relative près de la saturation et une concentration en saccharose et en azote élevée (Nguyen *et al.*, 2001). En condition naturelle, la photosynthèse contribue pour la majeure partie des besoins en carbone de la plante. En culture *in vitro*, le sucre exogène est ajouté au milieu comme source principale ou secondaire de carbone pour la plante afin de compenser partiellement l'effet négatif des conditions de culture sur le développement et pour maximiser la croissance des plantules *in vitro,* on parle alors de conditions de culture « photomixotrophique ». L'utilisation du sucre en micropropagation n'est pas sans désavantage puisqu'elle occasionne des perturbations métaboliques importantes. En conditions photomixotrophiques, il est généralement admis que la photosynthèse contribue de façon très limitée à la synthèse de composés carbonés chez les plantules. La présence de saccharose causerait une augmentation du contenu en hexose au niveau du cytosol et une diminution importante du recyclage du phosphate inorganique (Pi) qui seraient responsables de la diminution de l'activité de la Rubisco et conséquemment du taux de photosynthèse net (Desjardins, 1995). Plusieurs études ont démontrées l'impact négatif du saccharose sur l'activité photosynthétique des plantules notamment chez *Solanum tuberosum* (Wolf *et al.*, 1998), *Solanum lycopersicum* (Kubota *et al.*, 2001), *Coffea*

arabusta (Nguyen *et al.*, 1999), *Fragaria x ananassa* (Hdider & Desjardins, 1994) et *Cocos nucifera* (Fuentes *et al.*, 2005). Par ailleurs, on a observé un effet négatif du saccharose exogène sur l'expression de gènes impliqués dans la photosynthèse. Par exemple, l'ajout de glucose à des feuilles de *Spinacea oleracea* a causé la diminution de l'expression de la protéine D1 du photosystème II et du « light harvesting complex II » (Kilb *et al.*, 1996). L'utilisation de glucose dans la culture de *Triticum aestivum in vitro* a entraînée la répression de la fructose-1,6-biphosphatase, de la sedoheptulose-1,7-biphosphatase et d'une petite sous-unité de la Rubisco (Jones *et al.*, 1996). À l'opposé, d'autres données montrent que le sucre exogène a eu un effet positif sur l'activité photosynthétique de *Nicotiana tabacum* (Ticha *et al.*, 1998) et *Vitis vinifera* (Fila *et al.*, 1998). De plus, chez *Beta vulgaris*, l'ajout de sucre a provoqué une hausse importante de l'expression d'une petite sous-unité de la Rubisco, de la fructose-1,6-biphosphatase, et à un niveau plus faible, de la grosse sous-unité de la Rubisco (Kovtun & Daie, 1995). Les effets du sucre exogène sur le métabolisme carboné demeurent donc contradictoires et dépendent grandement des conditions expérimentales employées. L'effet du saccharose exogène sur le métabolisme des plantes *in vitro* serait beaucoup plus global qu'on le pensait et plusieurs études nous portent désormais à penser que ce facteur trophique aurait également un rôle majeur au niveau de la coordination et la régulation de la croissance.

L'ajout de saccharose au milieu de culture n'affecte pas seulement la photosynthèse, mais a un impact profond sur la distribution des composés carbonés dans la plante. Notamment, le milieu de culture entraîne un dérèglement de la relation normale source-puit (Desjardins *et al.*, 1993). En effet, les racines, qui agissent normalement comme des organes puits, agissent dans ces conditions comme des organes sources. On a récemment montré que le saccharose exogène modifierait le statut oxydatif des plantules. La présence de saccharose causerait une diminution de l'activité de deux superoxides dismutases (FeSOD et Cu/ZnSOD) localisées dans le chloroplaste et provoquerait l'augmentation de l'activité d'une autre (MnSOD) située dans la mitochondrie (Slesak *et al.*, 2006). Les superoxides dismutases (SOD) sont des enzymes clés impliquées dans la protection des cellules contre les espèces réactives de l'oxygène (ROS) et par conséquent, dans la protection contre les stress oxydatifs. Ces résultats seraient attribuables à l'inhibition de la photosynthèse et l'induction de la respiration par les sucres. Le lien étroit qui semble

exister entre le statut oxydatif des plantules et la morphogénèse suggère qu'une modification de l'activité des SOD pourrait jouer un rôle majeur dans le développement et l'acclimatation des plantules.

1.1.2 L'acclimatation des plantules *in vitro*

1.1.2.1 La problématique

L'environnement artificiel créé par la culture *in vitro* occasionne une modification importante de la physiologie, de la biochimie et de l'anatomie des plantules. Le phénotype caractéristique aux plantules *in vitro* est globalement incompatible avec l'environnement *ex vitro*. Ainsi, lors du transfert *ex vitro*, les plantules doivent rapidement modifier leur métabolisme pour s'adapter aux nouvelles conditions environnementales et assurer leur survie *ex vitro*.

Pour permettre une meilleure adaptation à l'environnement *ex vitro*, les plantules doivent passer par une période d'acclimatation (Pospisilova *et al.*, 1999). Au cours de cette étape transitoire, les plantules sont graduellement soumises à une hausse de la luminosité et une diminution de l'humidité ambiante. Cette étape permet ainsi aux plantules d'initier la production de nouvelles feuilles et racines adaptées aux nouvelles conditions environnementales. La croissance de nouveaux organes s'avère essentielle puisque les feuilles formées *in vitro* et tôt lors du transfert à l'acclimatation présentent une photosynthèse réduite et peuvent même présenter un bilan carboné négatif avec une transpiration et une respiration très élevées (Desjardins, 1995). Par exemple, une étude chez *Fragaria x ananassa* a montré que sitôt transférées *ex vitro*, les feuilles produites *in vitro* présentaient une faible activité photosynthétique et dégénéraient rapidement (Grout & Millam, 1985). Des résultats similaires sur l'effet du sucre sur la photosynthèse *ex vitro* des feuilles formées *in vitro* ont aussi été observées chez *Vitis vinifera* et *Castanea sativa* (Carvalho *et al.*, 2001). Malgré l'acclimatation progressive des plantules aux conditions *ex vitro*, un pourcentage élevé de plantules ne survivent pas au transfert. Ce problème est sans contredit l'un des plus sérieux rencontré par les laboratoires de micropropagation (Desjardins *et al.*, 1993) et résulte en une perte d'efficacité et une augmentation des coûts de production.

Plusieurs études ont évalué l'impact des conditions de culture *in vitro* sur le développement des plantules afin de mieux comprendre les problèmes observés lors de l'acclimatation. Au cours de ces études, on a constaté que les feuilles *in vitro* possédaient des caractéristiques anatomiques uniques. Par exemple, des feuilles de fraisier formées *in vitro* présentaient un parenchyme palissadique mince et de larges espaces cellulaires au niveau du mésophylle (Fabbri *et al.*, 1986). Plusieurs autres études ont constaté le même phénomène (Pospisilova *et al.*, 1999). De plus, lors du transfert *ex vitro*, les feuilles formées *in vitro* affichent globalement peu de signes de modification anatomique, mis à part une augmentation de leur taille, attribuable à l'expansion cellulaire (Donnelly & Vidaver, 1984). Parallèlement, cette étude a rapporté une diminution du nombre de trichomes et la présence de stomates dysfonctionnels et ouverts en permanence chez *Rubus idaeus*. Les plantules *in vitro* présentaient aussi une faible déposition de cuticule et de cire épicuticulaire, des feuilles minces et des stomates non-fonctionnels (Hazarika, 2006; Pospisilova *et al.*, 1999). Les multiples variations anatomiques observées chez les plantules *in vitro* les rendent sujettes au stress hydrique surtout lors de leur transfert *ex vitro* ce qui peut provoquer un dessèchement irréversible des plantules. De plus, les feuilles formées *in vitro* présentent une capacité limitée d'adaptation aux nouvelles conditions *ex vitro*. Les nouvelles feuilles formées *ex vitro*, mais initiées *in vitro*, présentent des caractères anatomiques intermédiaires, alors que les feuilles qui se forment par la suite affichent des caractéristiques se rapprochant plus de plantes produites en conditions naturelles.

Plusieurs recherches suggèrent que la présence de saccharose dans le milieu de croissance serait la principale cause du développement anormal de la photosynthèse *in vitro* et par conséquent, des difficultés d'acclimatation des plantules lorsque transférées en conditions *ex vitro* (Hdider & Desjardins, 1994). Cependant, toutes les études qui ont été menées sur le sujet ne permettent pas d'attribuer au sucre exogène un rôle aussi précis dans la survie des plantules lors du transfert *ex vitro*. Le retrait du saccharose ou la diminution de sa concentration dans le milieu de culture a eu un effet positif sur la croissance et l'acclimatation de *Hypericum perforatum* (Couceiro *et al.*, 2006), *Rehmannia glutinosa* (Cui *et al.*, 2000), *Eucalyptus camaldulensis* (Kirdmanee *et al.*, 1995) et *Rosa* sp. (Langford & Wainwright, 1987). À l'opposé, des conditions de culture photomixotrophiques ont eu un effet positif sur la croissance et l'acclimatation d'*Alocasia amazonica* (Jo *et al.*, 2009), de

Persea americana (Premkumar *et al.*, 2002) et de *Cocoa nucifera* (Fuentes *et al.*, 2005). Plus particulièrement, la présence de saccharose dans le milieu de culture a permis d'accroître le taux de croissance et l'activité photosynthétique chez des plantules de *Nicotiana tabacum ex vitro* (Haisel *et al.*, 2001; Hofman *et al.*, 2002; Kadlecek *et al.*, 2001). Sept jours après le transfert *ex vitro*, la présence de saccharose exogène *in vitro* a provoqué un allongement des entrenœuds de 2 à 4,4 fois supérieur à celui des plantules mises en croissance en absence de saccharose. Ces données contradictoires rendent difficile la connaissance exacte du rôle du sucre au cours de l'acclimatation. D'une part, l'effet bénéfique du sucre exogène sur la survie lors du transfert *ex vitro* est attribué à la présence accrue de réserve et par le fait même à une meilleure capacité à s'adapter aux conditions environnementales difficiles. D'autre part, il est suggéré que le sucre exogène aurait un impact négatif sur la survie des plantules en retardant le développement de l'autotrophie. Présentement, il existe un manque de données scientifiques sur la réponse physiologique des plantules *in vitro* au sucre exogène.

Certaines études montrent que l'environnement *in vitro* aurait un effet persistant sur la croissance et le développement des plantules (Haisel *et al.*, 2001; Kadlecek *et al.*, 2001). Après l'examen de l'anatomie et de la surface des feuilles *in vitro* des plantules de framboises et des nouvelles feuilles formées après une période d'acclimatation de 1 et 2 mois, des changements épigénétiques ont été observés (Donnelly & Vidaver, 1984). À l'échelle cellulaire, les effets épigénétiques se traduisent principalement par des modifications de la méthylation de l'ADN et des histones qui engendrent des changements de la conformation de la chromatine. De tels mécanismes épigénétiques permettent aux plantes de s'adapter à leur environnement en modifiant leur expression génique. Récemment, des modifications de la conformation de la chromatine ont été observées chez des cultures cellulaires de tabac dans un milieu de croissance *in vitro* (Law & Suttle, 2005). Les auteurs ont observé une diminution globale de la 5-méthylcytosine et une augmentation du taux de transcription de 300%. Une hypométhylation de l'ADN a aussi été observée chez des plantules de palmier à l'huile régénérées (Kubis *et al.*, 2003). Des études suggèrent maintenant que le sucre aurait un rôle dans l'induction de changements épigénétiques. Plus précisément, une protéine kinase SNF-1, un élément clé de la transduction des signaux du sucre, serait l'homologue d'une composante du complexe de

modification de l'ADN SWI-SNF chez la levure (Horvath *et al.*, 2003). On connaît encore peu les mécanismes qui induisent et permettent les variations de la méthylation de l'ADN, mais on peut considérer que l'environnement *in vitro* produit un effet épigénétique qui serait responsable, en partie, des problèmes d'acclimatation de certains plantules. Ce changement du patron de méthylation pourrait être un événement transmis au cours de quelques générations (Habu *et al.*, 2001).

1.1.2.1 Les stratégies

Trois stratégies ont été élaborées pour améliorer la survie des plantules lors du transfert en acclimatation. Premièrement, on a modifié les conditions de culture *in vitro* afin d'adapter les plantules aux conditions plus sévères présentent *ex vitro*. Cette stratégie repose principalement sur le meilleur développement de la photoautotrophie, la réduction de l'humidité relative à l'intérieur du contenant et la diminution du taux de transpiration des plantules. Deuxièmement, au cours de l'acclimatation comme telle, on évite la transpiration des plantules en utilisant des antitranspirants, et on tente d'accroître la photosynthèse en haussant le niveau de CO_2 atmosphérique et l'intensité lumineuse. Finalement, différentes études se sont attardées à stimuler le système de défense des plantules dans le but d'améliorer la capacité des plantules à tolérer les événements de stress *ex vitro*.

Pour pallier l'effet négatif du sucre exogène sur la photosynthèse et par ricochet, sur l'acclimatation, Kozai et son équipe (1997) ont mis au point un système de culture dit photoautotrophique. Ce système permet de diminuer les coûts de production et les risques de contamination du milieu de culture. Le retrait du sucre du milieu préconisé dans ce système de culture requiert des modifications importantes des paramètres de l'environnement *in vitro* conventionnel. Plus particulièrement, en absence de sucre exogène, la photosynthèse doit être assez élevé pour fournir une quantité de métabolites carbonés suffisante afin d'assurer une croissance soutenue et rapide des plantules. Par exemple, il est bien connu que la concentration en CO_2 à l'intérieur des contenants de culture subit une baisse rapide et drastique après le début de la photopériode (Nguyen *et al.*, 2001). Il faut alors favoriser le renouvellement de l'air ou assurer un enrichissement en CO_2 à l'intérieur des contenants de culture afin d'y maintenir une concentration en CO_2

acceptable pour soutenir la photosynthèse. De plus, pour obtenir une activité photosynthétique accrue, il est important de fournir un éclairage photosynthétique suffisant. C'est exactement ce qui a été observé chez *Coffea arabusta* (Nguyen *et al.*, 1999), *Lilium* sp.(Mei-Lan *et al.*, 2003), *Wrightia tomentosa* (Vyas & Purohit, 2003), des orchidées (Hahn & Paek, 2001) et *Solanum lycopersicum* (Kubota *et al.*, 2001). Selon certains, ce système de culture permettrait également de réduire le stress infligé aux plantules lors de l'acclimatation (Custodio *et al.*, 2004; Desjardins, 1995; Mei-Lan *et al.*, 2003).

On contrôle l'humidité dans les contenants de culture de plusieurs manières : en utilisant des dessiccants, en appliquant des composés huileux sur le milieu de culture, en ouvrant les contenants de culture dans des conditions de faible humidité, en ajoutant des membranes perméables pour assurer un meilleur échange d'air et en refroidissant la base de contenant afin de diminuer l'évaporation du milieu de culture (Hazarika, 2003). Dans bien des cas, la réduction de l'humidité relative *in vitro* s'est avérée efficace pour acclimater les plantules *in vitro* (Pospisilova *et al.*, 1999). Par exemple, chez *Chrysanthemum moriflorum* et *Beta vulgaris*, une diminution de l'humidité relative a causé une augmentation de la déposition de cire épicuticulaire, du nombre de trichomes et du fonctionnement des stomates, et conséquemment, une réduction des pertes hydriques (Ritchie *et al.*, 1991). Toutefois, la diminution de l'humidité entraîne un dessèchement prématuré non désiré du milieu de culture et une réduction du taux de multiplication.

D'autres stratégies, axées sur un meilleur contrôle de la transpiration des plantules, ont été étudiées autant *in vitro* qu'*ex vitro*. L'utilisation d'antitranspirants tel que la 6-benzylaminopurine, le glycérol, la paraffine et le latex et d'hormone tel que l'acide abscissique et l'acide indolebutyrique ont été efficaces pour améliorer l'acclimatation de certaines espèces (Nowak & Shulaev, 2003; Pospisilova *et al.*, 1999). Par exemple, l'ABA, un régulateur de croissance impliqué dans la fermeture des stomates en condition de stress, diminue de manière importante la conductance stomatique en culture *in vitro* et réduit le taux de transpiration lors du transfert *ex vitro* lorsqu'appliqué sur des plantules *in vitro* de *Nicotiana tabacum* en culture (Pospisilova, 1996). De plus, l'application d'ABA sur les plantules *ex vitro* a permis de diminuer la conductance stomatique des plants de *Nicotiana tabacum*, de réduire le stress hydrique et de favoriser la croissance lors de l'acclimatation

(Pospisilova *et al.*, 1998). L'application de l'inhibiteur de croissance paclobutrazol, une antigibbérelline, a aussi réduit la hauteur des plantules de *Prunus serotina* et diminué les pertes hydriques, ce qui a amélioré les taux de survie en acclimatation.

Dans la nature, la rhizosphère et les tissus des racines sont colonisés par une grande diversité de microorganismes. Les conditions stériles qui prévalent en culture *in vitro* privent les plantules de leurs effets parfois stimulants sur la croissance et par le fait même, sur l'induction des systèmes de défense de la plante. Plusieurs recherches se sont attardées à introduire différents microorganismes, principalement des bactéries et des mycorhizes, dans le milieu de culture *in vitro* avec un succès et des conséquences variables. Cette stratégie repose principalement sur l'amélioration de la croissance des plantules par l'ajout d'agent biologique permettant la stimulation des réactions de résistance systémique acquise (SAR), de la résistance systémique induite (ISR), l'allélopathie et l'amélioration du statut nutritionnel des plantules (Nowak & Shulaev, 2003). Globalement, l'induction du système de défense des plantules s'est avérés efficaces dans l'amélioration des relations hydriques, de la résistance aux pathogènes et de l'acclimatation.

1.2 Le saccharose

1.2.1 Assimilation du saccharose exogène par les plantules

La production de sucre par la photosynthèse est l'activité la plus fondamentale des plantes. L'ajout de sucre dans le milieu afin de compenser pour la faible activité photosynthétique des plantules est sans contredit une particularité inhérente à la culture *in vitro*. L'évolution du saccharose dans le milieu de culture et son prélèvement par la plante sont affectés par plusieurs paramètres (George, 1993). La stérilisation à l'autoclave entraîne une hydrolyse partielle du saccharose en glucose et en fructose. Le saccharose est aussi clivé par l'action d'invertases pariétales et/ou l'excrétion d'enzyme dans le milieu apoplastique. Pour plusieurs espèces, l'hydrolyse du disaccharide est majoritairement réalisée dans le milieu de culture avant son prélèvement (George, 1993). Les sucres sont assimilés par la plante par transport passif et actif et acheminés aux différents points de croissances de la plante. Chez la pomme de terre, des données récentes obtenues en employant du saccharose radioactif suggèrent que les plantules photomixotrophes

distribuent et métabolisent une proportion importante des sucres assimilés et excrètent une faible quantité de sucre dans le milieu de culture (Badr & Desjardins, 2007).

1.2.2 Mécanismes de régulation de l'expression génique par les sucres

La production de sucres par la photosynthèse est un processus vital des plantes. Le sucre n'agit pas seulement comme un facteur trophique mais il est aussi vecteur d'information sur l'état physiologique de la plante. La forme et la répartition des sucres dans la plante sont responsables de la coordination des signaux environnementaux qui déterminent la croissance et le développement. Chez les plantes, les sucres jouent un rôle important comme substrat de la respiration et en tant que composés structuraux et d'entreposage. L'effet des sucres sur l'expression génique, la croissance et le développement a longtemps été associé à son métabolisme et à la production d'énergie (Sheen *et al.*, 1999). À présent, plusieurs données montrent clairement l'existence d'un mécanisme de senseurs et de signaux de transduction distincts du rôle métabolique des sucres (Rolland *et al.*, 2002).

1.2.2.1 Voie de signalement des sucres

Malgré que le saccharose soit la forme majeure de sucre transloquée chez les plantes, des études montrent que se sont surtout les hexoses, glucose et fructose, qui sont impliqués dans la régulation génique. Le glucose et le fructose doivent préalablement être phosphorylés en glucose-6-phosphate et fructose-6-phosphate pour être utilisés dans le métabolisme de la plante. C'est l'hexokinase qui est responsable de cette réaction. Outre cette fonction, cette enzyme possède aussi un rôle au niveau de la régulation de l'expression génique (Fig. 1.1). Deux types principaux d'hexokinases ont été répertoriés chez les plantes, les types A et B. Le type A, localisé dans le stroma des chloroplastes, ne jouerait pas un rôle de senseurs d'hexoses. Par contre, le type B, associé à la membrane des mitochondries, serait étroitement associé à la réponse de la plante au sucre. L'utilisation de mutant a permis de caractériser la fonction signalétique des hexokinases. Par exemple, une hypersensibilité au sucre a été observé chez des plantules d'*Arabidopsis* surexprimant l'hexokinase 1 de type B (Xiao *et al.*, 2000). La complémentation d'un mutant de HXK1 par l'expression d'un isoforme d'HXK1 incapable de catalyser le transfert de groupement phosphate a permis de confirmer que le rôle signalétique de HXK1 n'est pas associé à sa

fonction catalytique (Moore *et al.*, 2003). Une faible quantité d'hexokinases, associés à la membrane des mitochondries, sont aussi transportés vers le noyau où ils forment un complexe avec une ATPase vacuolaire et une sous-unité du protéasome (RPT-5B) (Cho *et al.*, 2006). Les mécanismes impliqués dans le transport des hexokinases dans le noyau sont toujours inconnus, mais leur translocation dans le noyau permettrait la régulation de l'expression de plusieurs gènes, dont la protéine de liaison chlorophylle a/b (CAB) (Cho *et al.*, 2006).

Il existerait trois mécanismes de transduction des signaux reliés au glucose chez les plantes : hexokinase-dépendante, hexokinase-indépendante et glycolyse-dépendante ainsi qu'hexokinase-indépendante et métabolisme-indépendant (Sheen *et al.*, 1999). L'apport de 2-déoxyglucose et de mannose, deux molécules qui peuvent être phosphorylées par l'hexokinase, et de 3-O-méthylglucose et 6-déoxyglucose, des molécules qui ne peuvent être phosphorylées par l'hexokinase, à des protoplastes de maïs (*Zea mays*) et à des cellules en culture de concombre (*Cucumis sativus*) a permis de montrer que la phosphorylation était nécessaire pour que l'hexokinase régule l'expression de gènes liés à la photosynthèse et au cycle du glyoxylate (Graham *et al.*, 1994; Jang & Sheen, 1997). Cependant, la voie signalétique de l'hexokinase n'est pas essentielle pour la régulation génique en réponse au sucre de l'invertase et de la patatin (Ehness *et al.*, 1997; Roitsch, 1999); on parle alors de voie hexokinase-indépendante et glycolyse-dépendante. Par ailleurs, les données montrent l'existence d'un mécanisme de senseurs et de récepteurs pour le saccharose. Cette voie signalétique nommé hexokinase-indépendante et métabolisme-indépendante est distincte de celle des hexoses (Sheen *et al.*, 1999). Certains résultats suggèrent que la fonction signalétique du saccharose pourrait être en partie assurée par certains transporteurs de saccharose (SUT) (Fig. 1.1). Le transporteur SUT2 chez *Solanum lycopersicum* et *Arabidopsis* affiche une structure similaire aux transporteurs-senseurs de glucose SNF3 et RGT2 de la levure, ce qui suggère une implication possible de SUT2 comme senseur de saccharose (Barker *et al.*, 2000). Cependant, il existe encore peu de données sur le sujet et l'analyse de mutant sera nécessaire pour identifier les composantes du système de détection du niveau de saccharose chez les plantes.

1.2.2.2 Rôle de l'invertase et de la saccharose synthase

Afin d'initier la transmission des signaux du sucre par la voie signalétique hexokinase-dépendante, le saccharose doit être hydrolysé par l'invertase ou la saccharose synthase. Alors que la dégradation du saccharose par les invertases génère du glucose et du fructose, la saccharose synthase génère plutôt de l'UDP-glucose, ce qui produit ainsi deux fois plus d'hexoses (Koch, 2004). Le rôle de l'invertase et de la saccharose synthase dans la croissance et le développement est crucial. Une accumulation d'hexose favorise la division et l'expansion cellulaire (Roitsch & Gonzalez, 2004), et à l'inverse, un rapport hexose/saccharose plus faible favorise la différentiation et la maturation (Koch, 2004). Selon une hypothèse formulée par Koch (2004), l'invertase et la saccharose synthase contrôleraient le passage d'un stade de croissance à un autre. Dans ce cas, la formation de nouveaux organes-puits serait stimulée par l'activité invertasique (Fig. 1.1). Ultérieurement au cours du développement, l'activité invertasique diminuerait au profit d'une hausse de l'activité de la saccharose synthase, ce qui minimiserait la production de signal par les hexoses et stimulerait l'expression des gènes associés à l'entreposage des réserves et à la maturation des tissus.

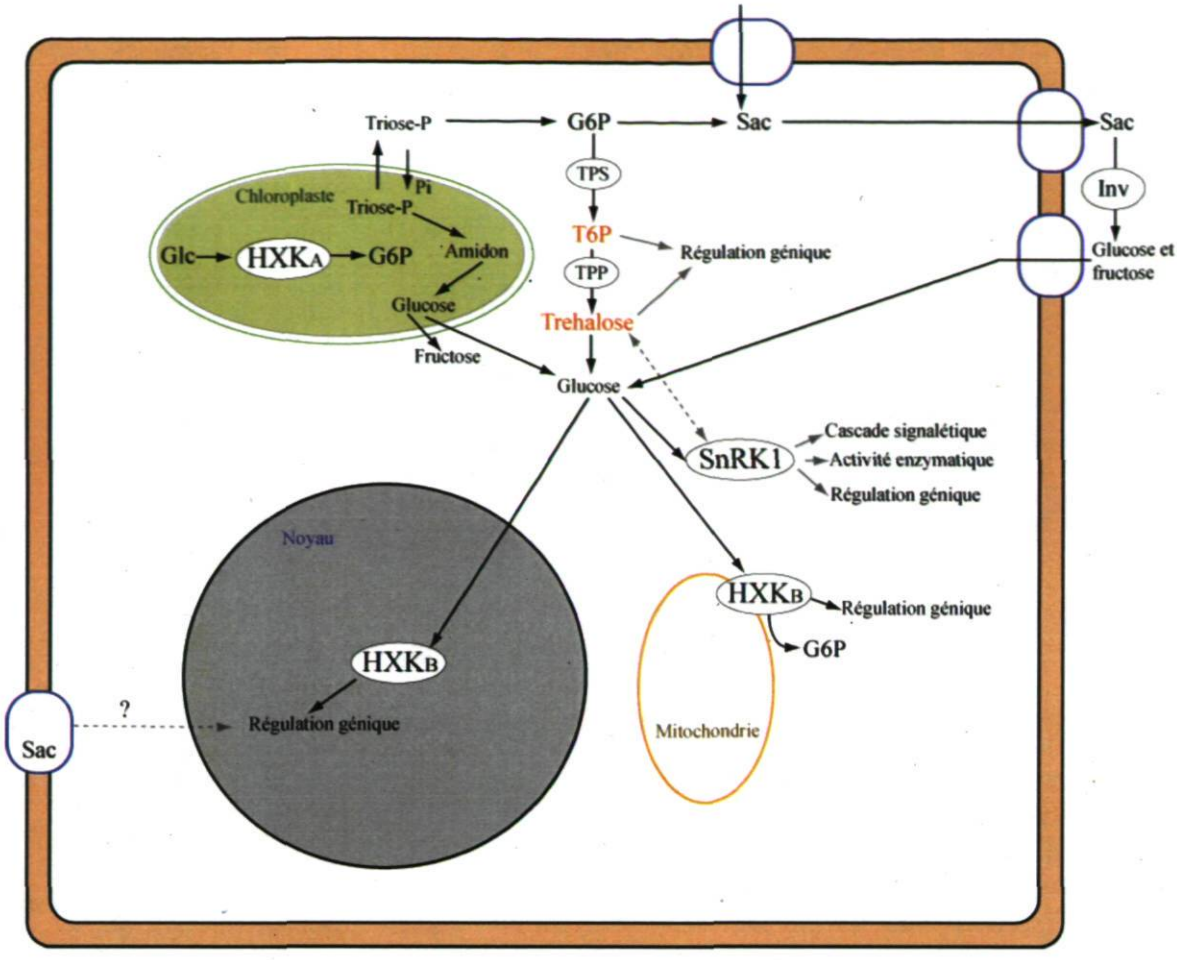

Figure 1.1 Représentation schématique des voies signalétiques induites par les sucres. L'hexokinase de type B (HXK_B) est principalement associée à la mitochondrie; elle participerait à la glycolyse, et serait aussi transportée dans le noyau. L'hexokinase de type A (HXK_A) est principalement située dans le chloroplaste. La protéine SnRK1 est impliquée dans la réponse à une carence en sucre et au stress. Le métabolisme du tréhalose serait une voie alternative pour le glucose-6-phosphate. Le tréhalose et le tréhalose-6-phosphate (T6P) sont des métabolites clés dans la régulation génique; ils permettraient la communication du statut en hydrates de carbones entre le cytosol et le chloroplaste et seraient responsable de la réponse au stress. Les Sac auraient vraisemblablement un rôle comme senseur du saccharose. Inv – Invertase pariétale; TPS – Tréhalose-6-phosphate synthase; TPP – Tréhalose-6-phosphate phosphatase; G6P – Glucose-6-phosphate; Glc – Glucose; T6P – Tréhalose-6-phosphate; SnRK1 – *sucrose non-fermenting related kinase 1*; HXK_A – hexokinase de type A; HXK_B – hexokinase de type B; Sac – Transporteur de saccharose

1.2.2.3 Le métabolisme du tréhalose

Le glucose-6-phosphate (G6P) produit par l'hexokinase peut être utilisé par plusieurs voies métaboliques telles que la glycolyse, la voie des pentoses phosphates et la synthèse d'amidon. Parallèlement, le G6P est aussi un substrat essentiel au métabolisme du

trehalose. En effet, la synthèse de tréhalose débute par la conversion du G6P et de l'UDP-glucose par l'entremise de la trehalose-6-phosphate synthase (TPS) (Fig. 1.1). Le trehalose-6-phosphate (T6P) est ensuite déphosphorylé par la trehalose-6-phosphate phosphatase (TPP) pour former du tréhalose. Le tréhalose et le T6P sont directement impliqués dans la régulation du métabolisme et du développement des plantes. En outre, le T6P est impliqué dans le contrôle de la photosynthèse, le développement, la paroi cellulaire, la biosynthèse d'amidon et la réponse aux stress. Le T6P agirait comme un signal permettant la communication du statut de la teneur en sucre du cytosol vers le chloroplaste (Paul, 2007). L'ajout de saccharose exogène a pour effet d'augmenter la production de T6P. L'accumulation de tréhalose permet de restreindre l'entrée du glucose dans la glycolyse lorsque ce dernier est trop abondant et qu'un déficit en ATP est engendré (Eastmond & Graham, 2003). Par ailleurs, une accumulation de tréhalose chez *Arabidopsis* se traduit par une augmentation importante du contenu en amidon. Le tréhalose aurait un effet sur l'expression et l'activité de l'ADP-glucose pyrophosphorylase (AGPase) et sur les activités respectives de la saccharose synthase et de l'invertase (Rolland *et al.*, 2002; Wingler *et al.*, 2000). À l'inverse, une carence en sucre stimule les diverses formes de la TPS, ce qui suggère que le T6P participe à la réponse au faible niveau de sucre (Thimm *et al.*, 2004; Wang *et al.*, 2007). Le T6P agirait aussi au niveau de l'activation redox de l'AGPase en aval de la protéine SnRK1 (Kolbe *et al.*, 2005). Le métabolisme du tréhalose serait en étroite collaboration avec la protéine SnRK1, impliquée dans la réponse au sucre (Rolland *et al.*, 2006). On remarque que plusieurs isoformes de la TPS possèdent des sites de phosphorylation spécifique à SnRK1. De plus, l'étude du profile d'expression d'*Arabidopsis* en présence de tréhalose exogène révèle une surexpression importante d'un gène qui appartient à la famille de SnRK1 (KIN11). Par contre, la surexpression de la KIN10 a entraîné la surexpression de plusieurs gènes TPS (Schluepmann *et al.*, 2004). L'application de tréhalose exogène chez des plantules d'*Arabidopsis* a causé la surexpression des gènes liés à la modification de la paroi cellulaire, à la biosynthèse d'acide gras, au métabolisme azoté, à la synthèse de facteurs de transcription, et de protéines de défense et à la voie signalétique de l'éthylène (Bae *et al.*, 2005a; Bae *et al.*, 2005b). Les mécanismes employés par le tréhalose et ses dérivés pour moduler l'expression génique,

l'activité enzymatique, l'activité photosynthétique et la répartition des composés carbonés restent encore nébuleux.

1.2.2.3 La protéine SnRK1

La protéine kinase SnRK1 (*sucrose non-fermenting 1-related kinase 1*) est une autre enzyme qui serait impliquée dans la fonction signalétique des sucres chez les plantes. Cette protéine jouerait un rôle important dans la détection du statut énergétique de la plante en situation de stress, de carence nutritionnelle et de faible luminosité (Baena-Gonzalez & Sheen, 2008). Elle permettrait, en outre, de synchroniser les différents métabolismes afin d'assurer l'établissement d'une réponse adaptative harmonieuse avec l'environnement, et favoriserait la croissance et le développement de la plante. En réponse aux changements du niveau de sucres intracellulaires, SnRK1 module l'activité enzymatique, la dégradation protéique et l'expression génique (Fig. 1.1). Des plants transgéniques d'*Arabidopsis* surexprimant le gène *Arabidopsis* kinase 10 (KIN10) affichent une meilleure tolérance et survie sous des conditions de faible luminosité alors que les plantes sauvages entrent rapidement dans un processus de sénescence (Baena-Gonzalez & Sheen, 2008). Jusqu'à tout récemment, peu de gènes régulés par la SnRK1 avaient été identifiés. Par exemple, l'expression de la saccharose synthase de la pomme de terre (*Solanum tuberosum*) et celle de l'alpha-amylase du blé (*Triticum aestivum*) sont activées et réprimées par la présence de sucre, respectivement, en lien avec l'expression de la SnRK1 (Laurie *et al.*, 2003; Purcell *et al.*, 1998). Baena-Gonzalez et al. (2007) rapportent que des plantules d'*Arabidopsis* surexprimant KIN10 stimule aussi des centaines de gènes impliqués dans le métabolisme du tréhalose et l'autophagie et impliqués dans la dégradation de la paroi cellulaire, de l'amidon, du saccharose, des acides aminés, des lipides et des protéines. De plus, la surexpression de KIN10 a induit plusieurs gènes codant pour des facteurs de transcription, des facteurs d'assemblage de la chromatine et des composantes du système de transduction des signaux carbonés. En somme, les protéines de la famille SnRK1 joueraient un rôle majeur dans les cascades signalétiques en réponse au manque de substrat énergétique et aux conditions de stress.

1.2.2 Régulation et interaction du sucre avec le métabolisme de la plante

Le sucre, en tant que molécule signalétique, est responsable de sa propre synthèse et de son propre catabolisme. Le changement du statut en hydrate de carbone des plantes, régulé par le système de signalement des sucres, est responsable de la modulation du développement et de l'adaptation des plantes aux conditions environnementales qui prévalent. Une première démonstration globale des impacts des sucres chez les plantes a été réalisée par Koch (1996). Cette auteure catégorise les gènes qui réponde au statut carboné en deux groupes : le premier groupe comporte les gènes qui sont surexprimés en présence d'une abondance de sucres alors que le second groupe est formé des gènes qui sont stimulés par les situations de « famines » (Koch, 1996). Une abondance de sucre induirait l'expression de gènes impliqués dans l'entreposage et l'utilisation du carbone. Par exemple, un niveau élevé de sucre foliaire enclencherait une rétroaction négative à l'égard de l'expression de gènes liés à la photosynthèse et induirait les gènes de la biosynthèse de l'amidon (Rook & Bevan, 2003). À l'opposé, un épuisement des sucres chez la plante favoriserait la stimulation des gènes de photosynthèse, la mobilisation des réserves et les processus d'exportation. Cependant, malgré l'avant-gardisme du scénario présenté par Koch (1996), les données récentes sur l'impact des sucres sur le métabolisme de la plante montrent que la réponse induite par la variation en sucres est plus complexe.

1.2.2.1 Régulation de l'expression génique

Plusieurs études récentes ont caractérisé l'impact du saccharose sur l'expression génique afin de mieux comprendre les changements métaboliques induits par les fluctuations en sucre. Ces études diffèrent quant aux traitements de sucres employés et aux systèmes de culture employés.

Ainsi, Contento *et al.* (2004) furent parmi les premiers à tester l'effet d'une carence en sucre sur l'expression génique de tissus de plantes. Pour ce faire, des cultures cellulaires d'*Arabidopsis* mis en présence de 2% de saccharose exogène ont ensuite été soumises à une carence en sucre pendant 24h et 48h. Ensuite, l'ARN messager des tissus a été hybridé sur une biopuce ATH1 de la compagnie « Affymetrix » comportant 22 750 fragments cibles. Ces auteurs ont observés qu'après 24h, des protéases vacuolaires impliquées dans l'autophagie étaient induites. Après 48h, plusieurs facteurs de transcription et plusieurs

gènes impliqués dans la transduction de signaux, dans la remobilisation des réserves, dans les défenses ont été surexprimées alors que les gènes impliqués dans la division cellulaire, la glycolyse, la synthèse de nucléotide, la synthèse d'acides aminés et de lipide, la transcription et la traduction étaient sous exprimées. Plus récemment, une étude similaire a été réalisée avec des cultures cellulaires de riz (*Oryza sativa*) (Wang *et al.*, 2007). Dans ce cas, les cultures de riz ont été transférées d'un milieu de culture contenant 3% de saccharose vers un milieu sans saccharose pendant 12h, 24h et 48h. Sous ces conditions, les données d'expression de plus de 21 495 fragments cibles ont permis de constater que plusieurs facteurs de transcription de la famille bZIP, NAC et WRKY, et plusieurs gènes impliqués dans le métabolisme du tréhalose, le transport des sucres et la dégradation des réserves étaient surexprimés. Par ailleurs, Price et al. (2004) ont ajouté du glucose à un taux de 2% pendant 24h à des plantules d'*Arabidopsis* mis en culture liquide agitée pendant 5 jours. Ces auteurs ont rapporté que plusieurs gènes impliqués dans le métabolisme carboné, la transduction de signaux, le transport, et le stress étaient régulés. Ces travaux ont aussi montré une plus faible expression de gènes impliqués dans la biosynthèse et la transduction de signaux de l'éthylène en présence de sucre exogène faisant ressortir un lien étroit entre le sucre et la voie signalétique de l'éthylène. Il importe de mentionner que la culture de cellule est un système relativement simple et versatile qui permet d'étudier rapidement l'impact des variations en sucre sur les tissus végétaux. Néanmoins, les données obtenues à l'aide de ce système sont difficilement applicable aux plantes entières étant donné que les cellules en culture sont des tissus non-photosynthétiques avec une communication cellulaire limitée. De plus, la réponse des plantules d'*Arabidopsis* en culture liquide agitée s'avère très sensible aux variations en sucre, passant de situation d'excès de sucre à une carence en sucre très rapidement (Osuna *et al.*, 2007). Pour étudier la réponse rapide aux sucres, Osuna *et al.* (2007) ont analysé les changements d'expression génique induits par une carence en sucre après 30 minutes et 3 heures. Dans cette expérience, des semences d'*Arabidopsis* ont été mises en culture liquide agitée en présence de saccharose (0,5%) pendant 7 jours, puis transférées pendant deux jours en absence de saccharose (0%) et finalement retransférées pendant un court laps de temps en présence de saccharose. Après 30 minutes sous ces dernières conditions, le saccharose a entraîné la surexpression de plus de 100 gènes, principalement des facteurs de transcriptions, des gènes de tréhalose-6-

phosphate synthases, des protéines d'ubiquitinations, une protéine d'autophagie et plusieurs glutarédoxines. Après 3 heures, des centaines de gènes impliqués dans le métabolisme carboné, dans la glycolyse, dans la respiration, dans la biosynthèse d'acides aminés, dans celle d'ADN et d'ARN, dans la biosynthèse de protéines et dans le repliement des protéines sont surexprimés. À l'inverse, les gènes impliqués dans le catabolisme des acides aminés et des lipides, dans la photosynthèse, dans la biogenèse des chloroplastes étaient quant à eux sousexprimés. En revanche, Thimm *et al.* (2004) ont étudié l'impact d'une carence en sucre en prolongeant la nuit de 6h chez des plantules d'*Arabidopsis* agées de 33 jours et cultivées en pleine terre. Les auteurs ont sélectionnées ce traitement afin de vérifier la coordination entre la synthèse et la dégradation des composés carbonés chez les plantes (Smith & Stitt, 2007). Selon ces auteurs, durant le jour, les photosynthétats seraient entreposés dans les feuilles principalement sous forme d'amidon, alors que durant la nuit, l'amidon serait dégradé et utilisé pour la croissance. Ce processus d'entreposage et d'utilisation des réserves serait hautement régulé et occasionnerait une hydrolyse presque totale des réserves en amidon à la fin de la nuit. La présence d'amidon en début de photopériode permettrait de mesurer la longueur de la nuit et ainsi ajuster la dégradation des sucres à leur accumulation durant la journée. Autrement dit, une prolongation subite de la nuit entraînerait conséquemment une carence en sucre durant une courte période de temps. Globalement, les résultats d'analyses du transcriptome portant sur l'effet d'une carence en sucre permettent maintenant de mieux comprendre la réponse physiologique et métabolique des plantes à la carence en sucre. En somme, la limitation énergétique engendre un stress métabolique qui enclenche l'arrêt de la croissance, l'activation des voies cataboliques et une diminution de l'activité des enzymes responsables de la biosynthèse.

1.2.2.2 Interaction avec le métabolisme azoté

L'azote est un élément minéral clé de la croissance des plantes et est souvent un facteur la limitant en condition naturelle (Paul & Foyer, 2001). Tout comme pour les sucres, le nitrate présente une double fonction : il agit d'une part à titre d'élément nutritif nécessaire à la formation des acides aminés et d'autre part, il agit comme molécule signalétique responsable de la régulation conjointe du métabolisme azoté et carboné. Plusieurs voies métaboliques sont stimulées par le nitrate, dont l'assimilation et la réduction du nitrate, l'assimilation de l'ammonium, la synthèse d'acides organiques alors que la

synthèse d'amidon est réprimée. Les mécanismes qui permettent de réagir à l'azote ne sont pas encore élucidés. Cependant, différentes études suggèrent que les plantes auraient la capacité de mesurer le contenu cellulaire en azote inorganique grâce aux signaux du nitrate. Pour sa part, le niveau en azote organique serait communiqué au reste de la plante grâce à l'ammonium, le glutamate ou la glutamine (Coruzzi & Zhou, 2001).

Malgré son rôle de premier plan, le métabolisme carboné n'agit pas seul dans la régulation de la croissance et du développement des plantes. Il est directement lié au métabolisme azoté. Toute modification de la quantité de composés carbonés entraîne simultanément des changements au niveau des différentes composantes du métabolisme azoté. Sur le plan physiologique, la production d'acides aminés à partir de l'assimilation du nitrate nécessite de l'ATP, des agents réducteurs et des composés carbonés provenant de la respiration, de la photosynthèse et de la glycolyse (Paul & Foyer, 2001). Le carbone requis pour l'assimilation du nitrate provient quant à lui du métabolisme des acides organiques et aurait deux rôles. D'une part, du malate est produit par l'entremise de la phosphoénolpyruvate carboxylase (PEPcase) et de la malate déshydrogénase comme contre-ion afin de prévenir une augmentation du pH cellulaire, notamment lors de l'assimilation du nitrate. D'autre part, de l'α-oxoglutarate est synthétisé par l'intermédiaire de la PEPcase, de la pyruvate kinase, de la citrate synthase, de la pyruvate déshydrogénase et de l'isocitrate déshydrogénase NADP-dépendente afin de fournir le carbone nécessaire à la production de l'ammonium (Stitt *et al.*, 2002).

L'implication du nitrate comme molécule signalétique se trouve à plusieurs niveaux du métabolisme de la plante. Un ajout de nitrate à des plantules d'*Arabidopsis thaliana* induit plusieurs gènes impliqués dans la synthèse et la maturation d'ARN, et dans l'activation de la synthèse d'acides aminés et de protéines (Czechowski *et al.*, 2004). De plus, une activation de la croissance et de l'expansion cellulaire a été observée alors que l'expression d'expansine et de protéines tonoplastiques étaient stimulée. Au cours de la même étude, un retrait de l'azote du milieu de culture a réprimé la majorité des gènes liés à la photosynthèse, à la synthèse de la chlorophylle et des protéines chloroplastiques (Czechowski *et al.*, 2004). Un faible niveau de nitrate stimule aussi l'expression de l'ADP-

glucose pyrophosphorylase ayant pour conséquence de favoriser le stockage des sucres en excès (Scheible *et al.*, 1997).

Plusieurs gènes régulés par les sucres sont aussi contrôlés par le nitrate; on pense en particulier à la nitrate réductase, à l'isocitrate déshydrogénase, à la pyruvate kinase, à la phosphoénolpyruvate carboxylase et à l'agpS2 (Scheible *et al.*, 1997). Par exemple, le saccharose a stimulé l'expression de la nitrate réductase lorsque le contenu de la feuille en 2-OG a été doublé par l'ajout de 2-OG exogène. Cependant, l'abondance des sucres a un effet direct sur la transcription des gènes du métabolisme azoté et est l'élément prédominant dans la régulation du métabolisme global. Ainsi, les sucres ont un effet stimulant sur l'expression des transporteurs de nitrate (NRT1 et NRT2), de la nitrate réductase, de la glutamine synthétase, de la pyruvate kinase, de la phosphoenolpyruvate carboxylase et de l'isocitrate déhydrogénase (Stitt, 1999; Stitt & Krapp, 1999). À l'opposé, l'asparagine synthétase (AS), responsable du stockage ou du transport de l'azote sous forme d'asparagine, est réprimée par un contenu élevé en sucre. De façon globale, les sucres causent une surexpression des gènes associés à la biosynthèse des acides aminés et une répression des gènes liés au catabolisme des acides aminés (Price *et al.*, 2004; Roessner-Tunali *et al.*, 2003). Des résultats similaires ont été observés sur l'activité enzymatique des protéines impliquées dans la voie de synthèse des acides aminés lorsque du saccharose exogène a été ajouté à des feuilles (Morcuende *et al.*, 1998). À l'inverse, une diminution de la quantité de sucre interne entraîne une interruption quasi totale de l'assimilation de l'azote, ou dans les cas les plus extrêmes, une inhibition de l'expression de la nitrate réductase et de l'accumulation de sa protéine. Ce mécanisme est enclenché à l'encontre des signaux du nitrate et du métabolisme azoté (Matt *et al.*, 2002; Stitt *et al.*, 2002). Cette situation exprime la dépendance du métabolisme azoté face au statut en composé carboné de la plante.

En somme, les travaux récents semblent montrer que le rôle signalétique des sucres serait prédominant sur l'azote dans la régulation du métabolisme azoté. Cependant, il est clair que l'azote intervient de façon importante dans la modulation de l'effet du glucose sur l'expression génique (Price *et al.*, 2004).

1.2.2.3 Interaction des sucres avec la prolifération cellulaire

De par leur nature et fonction, il n'est pas surprenant de constater l'impact des sucres sur le métabolisme des végétaux. Bien que les conséquences d'une variation du niveau des sucres soient bien connues, les mécanismes et les cascades métaboliques et signalétiques responsables de la réponse aux sucres le sont beaucoup moins. Toutefois, un certain nombre de données permettent actuellement de mieux comprendre l'impact du sucre sur le métabolisme des plantes.

La division cellulaire est un processus énergivore intimement lié au statut nutritionnel de la cellule. Dans cet ordre d'idée, des études ont établi le lien étroit qui unit la disponibilité en sucre à la division cellulaire. Plus précisément, des travaux chez *Arabidopsis* ont montré que l'expression des cyclines de type D, CycD2 et CycD3, requises pour la transition de la phase G1 à S, sont régulées par les sucres (Riou-Khamlichi *et al.*, 2000). Les deux CycD seraient impliqués dans le contrôle de la division cellulaire en tant que transmetteur du statut carboné de la cellule. Pour accomplir leur fonction, la CycD2 et la CycD3 agiraient ou non par l'entremise de la voie signalétique de l'hexokinase.

Par ailleurs, la régulation de la division cellulaire nécessite l'intervention de multiples autres processus métaboliques. Par exemple, la prolifération cellulaire requiert la synthèse de nouvelles protéines. Plus particulièrement, les protéines ribosomales, essentielles à la traduction, sont fortement régulées par le statut en sucre chez la plante (Osuna *et al.*, 2007; Thimm *et al.*, 2004). Récemment, l'induction des protéines ribosomales en réponse au sucre a été attribuée au gène de la nucléolin-1 (NUC-L1) chez *Arabidopsis* (Kojima *et al.*, 2007). L'ajout de 10 mM de glucose à une culture cellulaire d'*Arabidopsis* a causé une induction rapide du gène NUC-L1 et une stimulation de la croissance. L'étude d'un mutant d'*Arabidopsis* pour le gène NUC-L1 a permis de constater une réduction importante des protéines ribosomales. De plus, les mutants NUC-L1 présentent un phénotype similaire à plusieurs plantes mutantes déficientes pour certaines protéines ribosomales.

1.2.2.4 Interaction avec la production d'espèces réactives à l'oxygène et la défense

Les superoxydes et le peroxyde d'hydrogène, regroupé sous le terme « d'espèces réactive à l'oxygène (ROS)», sont des produits inévitables du métabolisme des plantes. En

effet, les ROS sont étroitement liés à la respiration mitochondriale, à la photosynthèse dans les chloroplastes et à la photorespiration dans les peroxysomes. Pour contrer la toxicité des ROS, les plantes font appel à différents processus antioxydatifs. Les superoxides dismutases (SOD) représentent la première ligne de défense responsable de la conversion des oxygènes réactifs en peroxyde d'hydrogène (H_2O_2). Par la suite, les cycles ascorbate-glutathione et glutathione peroxidase vont transformer le H_2O_2 en eau. En condition stable, la production et la destruction des ROS atteignent un équilibre. Toutefois, en réponse à différents facteurs environnementaux, biotiques ou abiotiques, le niveau intracellulaire de ROS peut augmenter rapidement et causer un stress oxydatif. Les ROS jouent ainsi un rôle majeur dans la cascade signalétique en réponse au stress.

La disponibilité en sucre est un important régulateur de l'activité respiratoire et photosynthétique, et par le fait même, un facteur déterminant dans la production de ROS à l'intérieur de la cellule. À l'opposé, les sucres sont aussi impliqués dans la régulation des processus antioxydants. En effet, le glucose procure le carbone nécessaire à la synthèse des caroténoïdes, de l'ascorbate et aux acides aminés impliqués dans la production de glutathione (Couée *et al.*, 2006). Par exemple, la présence de sucre exogène a causé une augmentation de la quantité d'ascorbate chez le brocoli (Nishikawa *et al.*, 2005). Il semble que l'induction des protéines de défenses par les ROS est accrue par la présence de sucre exogène ou par le niveau de sucres endogènes. En effet, le sucre exogène a un effet positif sur la régulation de protéines liées à la pathogenèse de la famille PR-2 (Thibaud *et al.*, 2004). De plus, le sucre exogène a permis d'augmenter la tolérance des plantules d'*Arabidopsis* à l'anoxie, vraisemblablement par la surexpression de plusieurs protéines de choc thermique (HSP) (Loreti *et al.*, 2005). La surexpression de différentes HSP a aussi été observée lors de l'ajout de glucose et de saccharose (Price *et al.*, 2004; Wang *et al.*, 2007).

Le rôle du sucre dans la régulation de la réponse au stress est de plus en plus probant. L'invertase pariétale a un rôle important dans le métabolisme en permettant la coordination de la photosynthèse, le métabolisme des puits et la réponse de défense en réponse aux sucres et aux stress (Roitsch & Gonzalez, 2004). Un bris mécanique, des éliciteurs, des pathogènes et différents stimuli biotiques entraînent l'induction des invertases pariétales (Roitsch *et al.*, 2003; Sinha *et al.*, 2002; Zhang *et al.*, 1996). Lors

d'une infection ou toutes autres situations de stress, une modification de l'activité et de la quantité d'invertases pariétales, et par conséquent du contenu en hexose, entraîne une induction du système de défense de la plante (Paul & Foyer, 2001).

1.3 Hypothèses et objectifs

Ce projet de doctorat s'articule autour de trois hypothèses principales :

1. L'expression génique des plantules en culture *in vitro* est très différente de celles ayant subi une acclimatation *ex vitro*. La croissance en conditions photomixotrophe stimule les gènes du métabolisme carboné (INV, SS) et azoté (NR, NiR, GS) et de réponses au stress et inhibe les gènes impliqués dans la photosynthèse, lorsqu'on les compare aux plantules *ex vitro*.
2. Le transfert des plantules des conditions *in vitro* vers le milieu *ex vitro* produit une cascade signalétique engendrée par le retrait du saccharose et stimule, à court terme, les gènes liés aux signaux de transduction (MAPK, protéine 14-3-3, CDPK, SnRK) et à la régulation du développement (HXK, TPS, facteur de transcription)
3. La présence de saccharose exogène dans le milieu MS (qui contient une forte teneur en N) interagit avec le métabolisme azoté et stimule la synthèse protéique et l'assimilation de l'azote.

Afin d'étudier et de vérifier ces hypothèses, les objectifs suivants ont été poursuivis :

1. Montrer que les conditions *in vitro* ont un effet marqué sur l'expression des gènes associés à la photosynthèse, aux mécanismes de stress, à la biosynthèse des sucres et des protéines par rapport aux plantules *ex vitro*.
2. Évaluer l'effet à court terme du retrait du saccharose du milieu de culture sur l'expression des gènes afin de mieux caractériser l'impact de son absence lors du transfert *ex vitro*.
3. Quantifier et identifier les variations de l'expression des protéines foliaires des plantules de tomate *in vitro* en présence et absence de saccharose exogène. Observer l'impact du saccharose exogène sur la synthèse et l'accumulation des protéines suite à un cycle de culture en conditions *in vitro*.

Chapitre 2 : Gene expression analysis reveals *in vitro* culture-induced regulation of primary metabolism and stress-related genes in tomato (*Solanum lycopersicum*) plantlet

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2.1 Résumé

Au cours de leur cycle de vie, les plantes sont soumises à des conditions environnementales changeantes qui requièrent constamment des réajustements métaboliques. En particulier, au cours de leur micropropagation, les plantules propagées *in vitro* sont confrontées à des conditions de croissance uniques de part la concentration d'azote et en saccharose exogène, la luminosité et la composition atmosphérique. Pour assurer une bonne reprise lors du transfert aux conditions *ex vitro*, les plantules *in vitro* doivent être acclimatées aux conditions naturelles non contrôlées. Dans le cadre de cette étude, l'impact de l'environnement *in vitro* sur l'expression de plus de 120 gènes de la tomate (*Solanum lycopersicum*) a été analysé grâce à une biopuce à ADN personnalisé et au PCR en temps réel. Lorsque comparées à des plantules *ex vitro*, les plantules *in vitro* âgées de 20 jours ont affiché une augmentation significative de l'expression des gènes impliqués dans la photosynthèse, le métabolisme de l'azote et la voie signalétique de l'acide salicylique, et une diminution des transcrits d'ARN des gènes associés au métabolisme carboné et à la voie signalétique de l'acide jasmonique. De plus, l'expression des gènes codant pour la synthèse d'enzymes impliquées dans le mécanisme de défense contre les dérivés réactifs de l'oxygène à l'intérieur des mitochondries, des chloroplastes et du cytosol était altérée *in vitro*. L'environnement *in vitro* a entraîné la surexpression de gènes impliqués dans la photosynthèse et l'assimilation de l'azote, la sousexpression des gènes liés à la force des puits et l'induction d'une réponse de stress chez les plantules de tomates. En somme, ces résultats suggèrent que la présence de saccharose exogène est un facteur environnemental clé qui cause la modification du profil cellulaire de la production des dérivés réactifs de l'oxygène, la modulation des gènes impliquées dans la défense et la physiologie anormale des plantules *in vitro*.

2.2 Abstract

During their lifespan, plants are submitted to changing environmental conditions that require constant metabolic adjustments. In particular, *in vitro* cultured plantlets are exposed, throughout micropropagation, to unique growth conditions characterized by distinct levels of nitrogen, exogenous sugar, light and atmosphere components. To resume growth upon transfer to *ex vitro* conditions, *in vitro* plantlets must acclimatize to the uncontrolled natural conditions. In this study, the impact of the *in vitro* environment on expression of about 120 genes of tomato (*Solanum lycopersicum*) plantlets was analysed using a custom designed DNA microarray and real-time PCR. When compared to *ex vitro* plantlets, 20 days old *in vitro* plantlets displayed a significantly increased expression of genes linked to photosynthesis, nitrogen metabolism and salicylic acid signaling, and decreased transcripts accumulation for genes associated with carbohydrate metabolism and jasmonic acid signaling. Also, the expression of genes coding for reactive oxygen scavenging (ROS) enzymes in mitochondria, chloroplasts and the cytosol were altered *in vitro*. The *in vitro* environment led to an increased expression of genes involved in photosynthesis and nitrogen assimilation, to a decreased expression of genes linked to sink strength, and to the induction of a specific stress response in tomato plantlets. Taken together, the results suggest that the presence of exogenous sugar is an important environmental cue that governs the modified cell ROS patterns, the modulation of defense-related genes and the abnormal physiology of *in vitro* plantlets.

Key words: acclimatization, gene expression, *in vitro* culture, *ex vitro* culture, primary metabolism, sucrose, *Solanum lycopersicum*, stress response

2.3 Introduction

During plant micropropagation, the transition from *in vitro* to *ex vitro* growth is a major bottleneck of the tissue culture process; the sudden change in growing conditions from a heavily controlled environment to the harsh and uncontrolled *ex vitro* conditions imposes severe stress on the plantlets and is responsible for high level of mortality. The *in vitro* environment is characterized by high relative humidity, high sugar content, high nitrogen, low CO_2, low light intensity, agar or liquid medium and limited gas exchange. These conditions have been associated with the abnormal physiology of *in vitro* plantlets and impose a strong adaptive pressure when these must eventually be transferred *ex vitro*, to natural conditions. The presence of high sucrose concentration in the culture media has been known to increase the cytosolic hexoses and decrease inorganic phosphate (P_i) recycling resulting in reduced Rubisco activity and reduced net photosynthesis (Desjardins, 1995). Under natural conditions, roots usually act as sink organs, however, *in vitro*, roots are submitted to high sucrose concentration that may impair their sink function and consequently, plant metabolism. The impact of *in vitro* exogenous sucrose on gene expression in *ex vitro* plantlets is not well established. In particular, information on gene regulation in plantlets cultured *in vitro* and upon their transfer *ex vitro* is still largely unknown.

In order to ensure high survival of plantlets upon transfer *ex vitro*, an acclimatization stage is needed to allow plants adaptation and resumption of growth. During this transition, plantlets are gradually submitted to increasing irradiance and CO_2 concentration, and decreasing relative humidity. Plantlet acclimatization is essential to allow renewed leaf and root production that are appropriately adapted to the *ex vitro* environment (Donnelly & Vidaver, 1984). The rapid initiation of new organs is crucial because *in vitro* formed leaves have a low photosynthetic rate and can rapidly become sinks *ex vitro* (Desjardins, 1995). Until now, a low photosynthetic rate and uncontrolled water loss have been considered as the main factors causing poor survival upon acclimatization (Hdider & Desjardins, 1994; Kirdmanee *et al.*, 1995; Premkumar *et al.*, 2002).

Many parameters of *in vitro* culture conditions are well known modulators of gene expression. Sugar feeding is considered a key determinant of the *in vitro* culture plantlets

phenotype (Desjardins *et al.*, 1993). As an energy source and as a signal molecule, sugars are important regulators of growth and development, photosynthesis and sink-source relationships (Koch, 2004; Paul & Foyer, 2001). In particular, sugars play a crucial role in cellular division, senescence, stress responses, and abscissic acid and ethylene biosynthesis and signaling. Long-term growth of plantlets in the presence of exogenous sucrose leads to altered metabolic fluxes and source/sink relationships. The plant's response to sugars is also highly determined by the nitrogen metabolism, which interacts with sugar metabolism and signaling at many levels (Coruzzi & Zhou, 2001; Morcuende *et al.*, 1998). Whereas soil nitrogen is usually a limiting factor, *in vitro* media generally contains very high concentrations of nitrate and ammonium, typically 60 mM in MS medium (Murashige & Skoog, 1962). Nitrate and N metabolites are key elements involved in gene regulation. Links with growth and development, primary metabolism, phytohormone synthesis and defense responses have been clearly demonstrated (Scheible *et al.*, 2004; Wang *et al.*, 2001). Nitrate inhibits starch synthesis and induces important modifications of the primary metabolism to increase organic acid synthesis and supply reducing power required for nitrogen assimilation (Fritz *et al.*, 2006). On the opposite, sugar starvation leads to a coordinated down-regulation of nitrogen assimilation while sugar abundance induces the expression of nitrate transporters, nitrate reductase, glutamine synthetase, pyruvate kinase and isocitrate dehydrogenase (Stitt, 1999; Stitt & Krapp, 1999).

While biotic stresses are not a concern in tissue culture, *in vitro* plantlets are submitted to a plethora of abiotic stresses. For instance, sucrose and nitrogen used at high concentration can impose a stress on tissues (Price *et al.*, 2004). In particular, variation of stomatal conductance (Fila *et al.*, 2006) and/or soluble sugars (Couée *et al.*, 2006) is directly linked to photosynthesis, photorespiration and mitochondrial respiration, the main sources of ROS (Mateo *et al.*, 2006). Imbalances in reactive oxygen species (ROS) production and detoxification are encountered in a multitude of environmental stresses, and now known to act as signaling cues leading to stress responses (Apel & Hirt, 2004). In addition, the presence of actively growing tissues and the poor diffusion of gases in the culture vessels have been reported to cause the accumulation of ethylene in the *in vitro* plantlet atmosphere (De Proft *et al.*, 1985; Mensualisodi *et al.*, 1992). Ethylene has numerous physiological roles in plant, but is also synthesized in response to various abiotic

stresses, such as wounding and anoxia, and initiates important changes in defense gene expression, particularly the activation of pathogenesis-related (PR) proteins (Rodrigo *et al.*, 1993; Tornero *et al.*, 1997). In cultured cells of tobacco (*Nicotiana tabacum*), for example, the accumulation of PR-5 proteins was demonstrated to be regulated by ethylene (Kitajima *et al.*, 1998). *In vitro* culture conditions are characterized by high concentration of salts, which is a well known abiotic stress activating wound-related genes including those encoding proteinase inhibitors in tomato (*Solanum lycopersicum)* plants (Dombrowski, 2003). Although it is strongly suspected that the *in vitro* culture environment leads to the induction of a defense-related responses in plantlets (Cassells & Curry, 2001), it has been poorly documented experimentally. The complex array of environmental factors that characterizes *in vitro* culture conditions must be considered when studying the physiological status of *in vitro* plantlets. Gene expression analysis using cDNA microarrays now offers the possibility to investigate and further elucidate the impact of the *in vitro* culture environment on plantlet responses. Therefore, the aim of this study was to compare *in vitro* cultured and *ex vitro* acclimatized plantlets using a custom made cDNA microarray consisting of 122 genes, specifically selected for there involvement in energy metabolism, carbon and nitrogen metabolism, growth and development, defense, protein folding and degradation, cellular function, transcription and signal transduction. Tomato was chosen as a model plant to study stress, carbon and nitrogen metabolism in an *in vitro* culture system.

2.4 Materiels and methods

2.4.1 Plant material and growth conditions

Tomato plantlets (*Solanum lycopersicum*) were micropropagated as described by Le et al. (2001). For the experiment, plantlets were grown *in vitro* in stacked doubled magenta vessels (Sigma, Oakville, ON, Canada). Each vessel contained a single plantlet. Axillary apices of single node cuttings were grown for 20 d on Murashige and Skoog (MS) medium containing 8% (w/v) bacto-agar (BD Diagnostic System, Sparks, MD, USA) and 3% (w/v) sucrose. In the growth room, plantlets were submitted to a 16 h photoperiod at 60 µmol photons m^{-2} s^{-1} PAR and a 23/20°C day/night temperature. After 20 d, half of the plantlets were harvested to form the *in vitro* pool, and half of the plantlets were transferred *ex vitro* in 10 cm pots for a 12 d acclimatization period. For the first 4 d, tomato plants were

maintained in an *Arabidopsis* ATC-10 chamber™ (Conviron, Winnipeg, MB, Canada) under high humidity condition, 16 h photoperiod at 175 µmol photons m^{-2} s^{-1} PAR and a 23/20°C day/night temperature. Plantlets were transferred to a greenhouse under natural daylight (August) at Laval University (Quebec City, Canada, 46°48' N) for the remaining 8 d in order to complete the acclimatization process and harvested. At the end of the *ex vitro* culture stage, the second leaf corresponded to a newly *ex vitro* formed leaf. The second leaf from each plantlet were immediately and individually frozen in liquid nitrogen and stored at -80°C before further grinding.

2.4.2 Microarray preparation

A custom made cDNA microarray was developed for the experiments. The array included genes specific probes associated with photosynthesis, carbon and nitrogen metabolism, signal transduction, stress and defense mechanisms, and housekeeping functions. Primers for 120 genes were chosen using the software Primer3 (Rozen & Skaletsky, 2000). Genes or EST sequences were accessed via the National Center for Biotechnology Institute (NCBI). PCR product sizes were ranging from 200 to 800 bp. The PCR reactions were performed with the Expand high fidelity (Roche Diagnostics, Laval, QC, Canada) and PCR TGradient Thermocycler (Biometra, Goettingen, Germany). Each PCR product was purified with Montage PCR filter units (Millipore, Billerica, MA) and subject to automatic sequencing (SUCOF, QC, Canada) to confirm the authenticity of the amplified fragment. The amplicons were arrayed on GAPS II coated slides (Corning, Lowell, MA) using the VersArray ChipWriter Pro (Bio-Rad, Mississauga, ON, Canada). All genes on the array were printed in at least six replicates to take into consideration possible technical variations. The SpotReport Alien cDNA Array Validation System (Stratagene, La Jolla, CA), consisting of two artificial Alien sequences, was printed on the array. Print quality and detection sensitivity of the arrays was monitored by the addition of these external and negative controls. A 700-pb fragment of the green fluorescent protein (GFP) was also added to the biochip as a positive control. A Terminal Transferase dye assay was performed in order to ensure quality of the slide.

2.4.3 RNA extraction and cDNA synthesis

Total RNA was isolated using the Plant RNA Reagent kit (Invitrogen, Burlington, ON, Canada). RNA concentration was measured using an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). Possible contamination was assessed by monitoring of the 260/280 nm OD ratio. Reverse transcription of 40 µg of total RNA was performed with the Superscript III kit (Invitrogen). Aminoallyl-dUTP (Applied Biosystems, Streetsville, ON, Canada) was added to the cDNA synthesis reaction to allow further indirect labelling. RNA of the hybrid cDNA-RNA was hydrolyzed by the addition of 1M NaOH and 15 min heating at 65°C. The reaction was neutralized by the addition of HCL 1M. A final purification step was realized using the QIAquick PCR purification kit (Qiagen, Mississauga, ON, Canada).

2.4.4 Probe labelling and hybridization

Three biological replicates consisting of three individual plantlets grown in different vessels (*in vitro*) or pots (*ex vitro*) were used for the competitive hybridization. To reduce possible biases due to differences in the properties of the dyes, two technical replicates consisting of dye swap were carried. The complete experiment required six microarrays. Samples were labelled using AlexaFluor 555 or 647 reactive dye packs (Invitrogen), according to the manufacturer. The cDNA labelling step was performed for a 3-h period. Following a purification step using the Qiaquick PCR purification kit (Qiagen), 600 ng of labelled cDNA of each probe were pooled for hybridization. Preliminary tests indicated this amount of cDNA to confer high level and range of intensity for most spots. The SlideHyb buffer #1 (Applied Biosystems) was added to the labelled cDNA. Hybridization was performed using a SlideBooster from Advalytix AG (Brunnthal, Germany). To this end, a lifterSlip (Erie Scientific Company, Partsmouth, NH) was laid down on the array and the hybridization solution injected. These hybridization chambers facilitate the migration of cDNA by the emission of micro-impulsions under the glass slide. This technology greatly enhances hybridization efficiency. After 17 h of hybridization at 55°C, slides were washed in a low and high stringency buffer as mentioned in the SlideHyb buffer #1 protocol (Applied Biosystems).

2.4.5 Data analysis

The slides were scanned using a VersArray ChipReader (BioRad, Mississauga, ON, Canada). Digital images were acquired with the ChipReader 3.1 software (Bio-Rad). Data image were transferred to the Array-Pro Analyzer v.4.5 software (Media Cybernetics, Silver Spring, MD, USA) to detect the intensity of each spot on the array and for background subtraction using the local ring method. Net intensity data were $\log_2$ transformed. The data were normalized using the global Loess method included in the Microarray Analysis of Variance (MAANOVA) software. Normalization was performed in order to consider possible intensity-dependent variations in dye bias. Statistical analysis was accomplished using the F-test of the R/maanova package (Wu *et al.*, 2003), which works under the R programming environment. The false discovery rate (FDR) procedure was applied to the p-values in order to minimize the number of false positives associated to multiple testing. Differentially expressed genes were selected according to the adjusted p-values. Only genes with an adjusted p-value under 0.01 were kept for further analysis. In addition, the fold change in expression between the two treatments had to be higher than 1.5 to be considered. Microarray data were placed in the NCBI public repository "Gene Expression Omnibus (GEO)" (accession no. GSE10153, www.ncbi.nlm.nih.gov/geo).

2.4.6 Validation and gene expression analysis by real-time quantitative PCR

Real-time quantitative PCR (RT-PCR) was used to validate the results obtained with cDNA microarrays and to further investigate the impact of culture condition on gene expression. To proceed, 2 µg of total RNA was treated with RNase free DNase (Roche). Complementary DNA was synthesized with Omniscript RT kit (Qiagen) according to the manufacturer's protocol using oligo $d(T)_{18}$. Quant-it RiboGreen RNA Reagent and Kit (Invitrogen) was used to calculate cDNA concentration as described by Libus and Storchova (2006). Prior to RT-PCR, primers were designed using Primer3 (Rozen & Skaletsky, 2000). Each primer was subjected to the Basic local alignment search (BLAST) to identify possible unspecific homology with other tomato transcripts. NCBI accession numbers for the genes studied for microarray validation were: AY240926 (Asparagine synthetase (*AS*)), AW034391 (Glutathione reductase (*GR*)), AJ295638 (Cathepsin D inhibitor (*CDI*)) and U89256 (DNA-binding protein pti5 (*PTI5*)). Expression analysis was

also conducted for the three other genes: X14041 (chloroplastic Cu-ZnSOD (Cu-Zn*SOD*$_{chl}$)), X14040 (cytosolic Cu-ZnSOD (Cu-Zn*SOD*$_{cyt}$)) and AI482836 (mitochondrial MnSOD (Mn*SOD*)). RT-PCR reactions were carried out in glass capillaries with a Light Cycler™ Real-Time quantitative PCR using Fast Start DNA SYBR® Green (Roche). A standard curve, consisting of five dilutions of the original template, was used for the absolute quantification of mRNA transcript for each gene. Gene identity was confirmed by DNA sequencing. Five biological replicates consisting of three individual plantlets grown in different vessels (*in vitro*) or pots (*ex vitro*) were used for RT-PCR assays. Expression data of the gene studied were normalized on the basis of cDNA concentration of each sample. Data analysis was accomplished using the SAS software (Sas institute, Cary, NC).

2.5 Results

Micropropagation of tomato plantlets is achieved by explanting the axilliary bud from a node cutting and subculturing it on MS medium containing 3% (w/v) sucrose. For the first week following subculture, the new apex primarily initiated root followed by the development of new leaves. During the entire *in vitro* stage, plantlets were cultured photomixotrophically, i.e. relying on exogenous sucrose and photosynthesis for their carbon needs. After 20 d, tomato plantlets had two to three leaves formed *in vitro* and were ready to be transferred *ex vitro*. After a 12 d acclimatization period, plantlets had established new roots and leaves adapted to the *ex vitro* environment. Upon transfer, *in vitro* formed leaves underwent a disorganized expansion, or simply senesced. Even though there were important differences between both environments, leaves harvested from *in vitro* and *ex vitro* plantlets were both developmentally in the expansion stage.

The impact of *in vitro* culture conditions on gene expression was investigated by performing a competitive hybridization of mRNA transcripts extracted from tomato leaf tissues grown *in vitro* or *ex vitro*. For this purpose, a cDNA microarray was built in order to monitor the simultaneous expression patterns of some 122 genes (see supplementary table in Annexe 1 for complete list of metabolically-classified genes). Following analysis, genes (adjusted p-value ≤ 0.01) displaying a 1.5-fold change in expression were selected for interpretation in order to maximise the capacity to identify trends in gene expression. Using the criteria chosen for significance, a total of 26 genes were considered for further analysis

(Table 2.1). Some genes (adjusted p-value ≤ 0.05) that did not fulfill our criteria, such as those for the sucrose-phosphate synthase (*SPS*) and allene oxide synthase (*AOS*), were still included in the interpretation to reinforce and facilitate the understanding of the *in vitro* plantlet metabolism. Real-time quantitative PCR (RT-PCR) was used to further investigate the results obtained with the cDNA microarray platform. Results indicated that *in vitro* culture conditions were associated with specific expression patterns for genes associated to photosynthesis, defense, and carbon and nitrogen metabolism when compared to *ex vitro* plantlets.

Table 2.1: Number of regulated genes for each functional categories present on the cDNA microarray

Functional category	Number of genes		
	in the category	Up-regulated[a]	Down-regulated[a]
Defense-related	30	6	5
Signal transduction and transcription	10	0	0
Sucrose metabolism	10	0	3
Cellular respiration	12	0	3
Energy metabolism	10	3	0
Nitrogen metabolism	6	4	1
Growth and development	6	0	1
Protein folding and degradation	28	0	0
Housekeeping	6	0	0
Others	4	0	0
Total	122	13	13

[a]Gene with an adjusted p-value under 0.01 and a fold change higher than 1.5 were designated as "regulated". Gene up-regulated and down-regulated under *in vitro* culture conditions with respect to *ex vitro* plantlets.

2.5.1 Lower expression of carbohydrate metabolism associated genes *in vitro*

Not surprisingly, differences in gene expression for sucrose metabolism and cellular respiration were observed between *in vitro* and *ex vitro* plantlets. For instance, a sucrose transporter, *SUT4*, was down-regulated in *in vitro* tomato plantlets (Table 2.2). *In planta*, sucrose transport and unloading is achieved through sucrose transporters localized mainly in enucleate sieve elements and unloading of sucrose in sink cell include the irreversible cleavage of sucrose to glucose and fructose by invertase. The results indicate that an extracellular invertase, *LIN5* (EC 3.2.1.26), had a 4-fold lower expression rate in *in vitro* than *ex vitro* plantlets (Table 2.2). Moreover, the tomato hexokinase 1 (*HXK1*) mRNA was

significantly down-regulated *in vitro* (Table 2.2). In a less pronounced but similar fashion, mitochondrial ATP synthase β subunit (ATPase β) (EC 3.6.3.14) mRNA was significantly down-regulated under *in vitro* culture conditions (Table 2.2). Mitochondrial ATP synthase is responsible for ATP production in the mitochondrial matrix using a proton gradient. More precisely, the β subunit is included in the F1 unit of ATP synthase and is known to act as a nucleotide binder. Following the same trend, *AGPL1*, which encodes an ADP-glucose pyrophosphorylase (EC 2.7.7.27) involved in starch synthesis in guard cells in tomato (Li *et al.*, 2002), presented a 2.5-fold lower expression level in *in vitro* plantlets (Table 2.2).

Table 2.2: Transcripts identified with cDNA microarrays showing down-regulation in tomato plantlets under *in vitro* culture conditions, as compared to plantlets acclimatized *ex vitro*.

Genbank accession no.	Gene identity	Microarray expression ratio[a]
	Carbohydrate and energy metabolism	
AJ272304	Beta-fructofuranosidase (*LIN5*)	4.0
AJ401153	Hexokinase (*HXK1*)	3.6
U88089	ADP-glucose pyrophosphorylase large subunit 1 (*AGPL1*)	2.5
L27509	NADP-dependent malic enzyme (*ME1*)	1.9
AF176950	Sucrose transporter (*SUT4*)	1.7
AW031780	ATP synthase beta subunit, mitochondrial precursor	1.6
	Nitrogen metabolism	
X92853	Nitrate transporter 1.1 gene (*NRT1.1*)	3.1
	Defense	
AJ295638	CathDInh gene for Cathepsin D Inhibitor (*CDI*)	5.1
X73986	Cathepsin D inhibitor protein (*CDI*)	4.6
U37839	Lipoxygenase (*LOXC*)	2.8
U50151	Leucine aminopeptidase (*LAP-A*)	2.5
X98929	Serine protease (*SBT1*)	2.2
	Growth and development	
AJ270960	Expansin 18 gene (*EXP18*)	1.7

[a] Statistical analysis was done by using the F-test of the R/maanova package (Wu *et al.*, 2002). Only genes showing an adjusted p-value under 0.01 and a fold change higher than 1.5 are presented.

2.5.2 Higher expression of specific components of the photosynthetic apparatus *in vitro*

Modification of photosynthetic rate and efficiency is a major issue in micropropagation. The microarray analysis revealed the induction of genes associated with the photosynthetic apparatus proteins and reactions under *in vitro* culture conditions.

Rubisco activase (*RCA*) expression was 5.5-fold higher in micropropagated than acclimatized plantlets (Table 2.3). The same trend was observed for two proteins associated with the photosystems. Photosystem I reaction centre subunit x precursor (*PSAK*) transcripts in acclimatized plantlets was only present at 40% of the level found in *in vitro* plantlets (Table 2.3). A 10-kDa polypeptide precursor of photosystem II, *PSBR*, was also up-regulated under *in vitro* culture conditions (Table 2.3). PSBR is linked to the oxygen evolving complex of photosystem II (PSII). It has been shown recently to be crucial to photosynthetic water hydrolysis and to the binding of some other subunits of PSII, *PSBP* and *PSBQ* (Suorsa *et al.*, 2006). Inversely, a significantly lower amount of RNA transcripts for the chloroplastic NADP-dependent malic enzyme (NADP-ME) (EC 1.1.1.40) was also observed in *in vitro* plantlets compared to *ex vitro* plantlets (Table 2.2).

2.5.3 Increase in transcripts of gene associated with nitrogen metabolism

It was found that four genes involved in nitrogen metabolism were up-regulated *in vitro*. The nitrite reductase (*NIR*) (EC 1.7.7.1) gene, involved in nitrite conversion to ammonium, was up-regulated in plantlets grown *in vitro* for 20 d (Table 2.3). We also observed an up-regulation of genes associated with the GS/GOGAT cycle. mRNAs for glutamine synthetase 2 (*GS2*) (EC 6.3.1.2) and glutamate synthase (*GOGAT*) (EC 1.4.1.14) were induced 2.2- and 2.1-folds, respectively, by the *in vitro* culture conditions (Table 2.3). One of the genes exhibiting the highest increase of its transcripts under *in vitro* conditions was the 7.1-fold up-regulated asparagine synthetase (*AS*) (EC 6.3.5.4) mRNA (Table 2.3). In addition to asparagine, the movement and distribution of nitrate through the plant is regulated by transporters with different affinity for nitrate. The nitrate transporter *NRT1.1*, a member of the family of low affinity high capacity transport system, was repressed by *in vitro* culture conditions (Table 2.2). *NRT1.1* was the only gene associated with the nitrogen metabolism that was significantly down-regulated *in vitro*. Thus, differential regulation of pivotal intermediate of the nitrogen metabolism upon transfer from an *in vitro* to an *ex vitro* environment could be linked to the difference in nitrogen concentration.

Table 2.3: Transcripts identified with cDNA microarrays showing up-regulation in tomato plantlets under *in vitro* culture conditions, as compared to plantlets acclimatized *ex vitro*.

Genbank accession no.	Gene identity	Microarray expression ratio[a]
	Energy metabolism	
AI484371	Rubisco activase (*RCA*)	5.5
BF052204	Photosystem I reaction centre subunit x precursor (*PSAK*)	2.5
AW037270	Photosystem II 10 kDa polypeptide precursor (*PSBR*)	1.8
	Nitrogen metabolism	
AY240926	Asparagine synthetase (*AS1*)	7.1
BG791272	Nitrite reductase (*NIR*)	4.9
U15059	Chloroplast glutamine synthetase (*GS*)	2.2
AW032148	Glutamate synthase (NADH) precursor (*GOGAT*)	2.1
	Defense-related	
AW218786	Pathogenesis-related, osmotin-like protein (*PR P23*)	14.4
AW035574	Pathogenesis-related leaf protein 6 precursor (*P6*)	4.5
M69247	Pathogenesis-related protein (*P4*)	4.2
Y08844	Pathogenesis-related protein (*PR1a2*)	4.1
AJ251882	Ascorbate peroxidase (*APX*)	2.3
AW034391	Glutathione reductase (*GR*)	1.7

[a] Statistical analysis was done by using the F-test of the R/maanova package (Wu *et al.*, 2002). Only genes showing an adjusted p-value under 0.01 and a fold change higher than 1.5 are presented.

2.5.4 Up-regulation of mRNA transcripts involved in pathogenesis and oxidative stress

Surprisingly, more than one third of the genes showing significant variations of their expression in *in vitro* plantlets were related to defense. The close homologues PR proteins *P4* and *P6*, also called Pr-1a and Pr-1b, presented a significantly higher transcript level under the *in vitro* environment compared to the *ex vitro* acclimatization (Table 2.3). Expression analysis revealed a strong induction of *PR1a2* protein mRNA in *in vitro* plantlets (Table 2.3). Among the genes regulated in this experiment, the pathogenesis-related osmotin-like protein (*PRP23*) was the gene exhibiting the highest fold change (Table 2.3). Compared to *in vitro* plantlets, transcript levels for *P23 ex vitro* was low or near the detection limit. Clearly, the increase expression of defense-related genes suggests that *in vitro* conditions induced defense-related responses.

In the chloroplast, the removal of toxic superoxides and hydrogen peroxides, and their conversion to water, is mostly achieved by superoxide dismutase (SOD), ascorbate

peroxydase (APX) and glutathione reductase (GR). In this experiment, the expression of tomato *APX* and *GR* targeted to the chloroplast were higher under *in vitro* culture conditions (Table 2.3). The chloroplastic Cu-Zn*SOD* gene presented a significantly lower level of expression when plantlets were grown *in vitro* (Fig. 2.1A). Its close homologue, the cytosolic SOD (Cu-ZnSOD_{cyt}), was also down-regulated in *in vitro* plantlets (Fig. 2.1B). The production of ROS also occurs through the complexes I and III of the electron transport chain in mitochondria. The expression analysis by RT-PCR revealed that mitochondrial targeted Mn*SOD*, localized in the matrix, was up-regulated in plantlets grown under the *in vitro* environment (Fig. 2.1C).

2.5.5 Down-regulation of jasmonate biosynthesis and proteinase inhibitor transcripts

The microarray analysis revealed that several genes associated with stress events are down-regulated by *in vitro* culture conditions. Transcript levels for two genes encoding key enzymes of the octadecanoid/JA pathway were lower in *in vitro* plantlets. These genes, encoding a chloroplast lipoxygenase C (*LOXC*) (EC 1.13.11.12) (Table 2.2) and an *AOS* (EC 4.2.1.92) (data not shown), were down-regulated 2.8- and 2-fold *in vitro*, respectively. The data showed a striking 4- to 5-fold reduction in the level of transcripts of cathepsin D inhibitor (*CDI*) *in vitro* (Table 2.2). A serine protease gene (*SBT1*) (EC 3.4.21.14) from the subtilase family as well as a leucine aminopeptidases, *LAP-A* (EC 3.4.11.1), followed the same expression trend *in vitro* and were down-regulated 2.2- and 2.5 folds (Table 2.2).

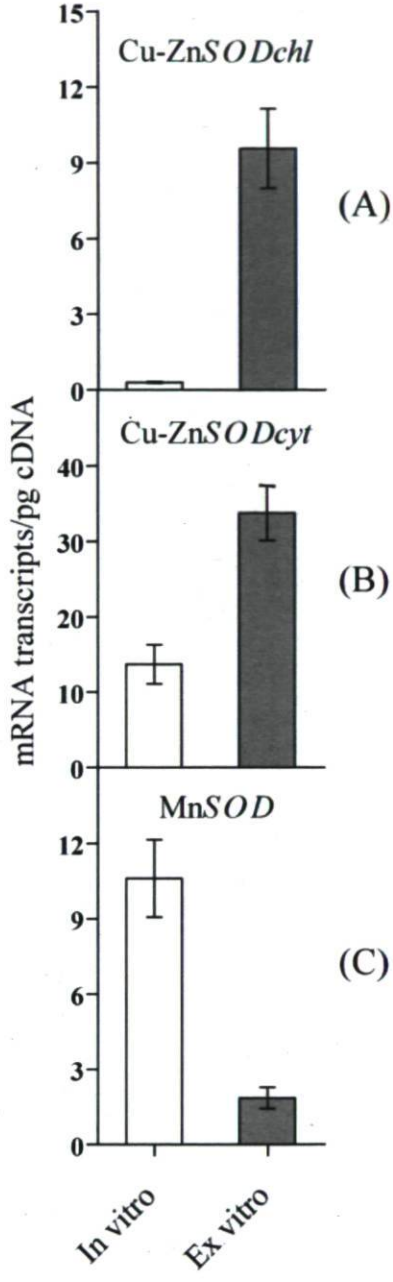

Figure 2.1 Changes in mRNA transcripts level of three superoxide dismutase (SOD). (A) chloroplastic Cu-ZnSOD (X14041) (B) a cytosolic Cu-ZnSOD (X14040) and (C) a mitochondrial MnSOD (AI482836) gene expression was monitored by RT PCR. Total RNA was extracted from plantlets cultured *in vitro* for 20 d (*in vitro*) and compared with plantlets cultured for 20 d *in vitro* and transferred *ex vitro* for 12 d (*ex vitro*). Data represent mean expression of five measurement ± SE. Expression data were normalized on the basis of cDNA amount as described by Libus and Storchova (2006). Result for Cu-ZnSODchl, Cu-ZnSODcyt and MnSOD were statistically significant at $p \leq 0.05$.

2.5.6 Growth and development defects *in vitro*

In our study, the expression of only one gene specifically related to growth and development was modulated. A tomato expansin gene (*EXP18*) was repressed by the *in vitro* environment (Table 2.2). Expansins are extracellular proteins known to be involved in cell wall expansion. High *EXP18* expression has been closely related with meristematic activity such as apical meristem, leaf primordia, vascular tissue of young stem and flowers (Reinhardt *et al.*, 1998).

2.5.7 Validation of microarray expression data

The expression of four genes, namely *AS1*, *GR*, *CDI* and DNA-binding protein Pti5 (*PTI5*) was verified by RT-PCR to validate the data obtained with the microarray analysis (Fig. 2.2). These genes were selected for RT-PCR analysis because they were respectively up-regulated, slightly up-regulated, down-regulated or unregulated *in vitro* (Table 2.2 and 2.3). For the three genes significantly regulated, microarray results were confirmed by the RT-PCR analysis, although the changes were obviously greater with the RT-PCR results (Fig. 2.2A, B, and C). By contrast, no significant difference was observed for *PTI5*, in agreement with the microarray data.

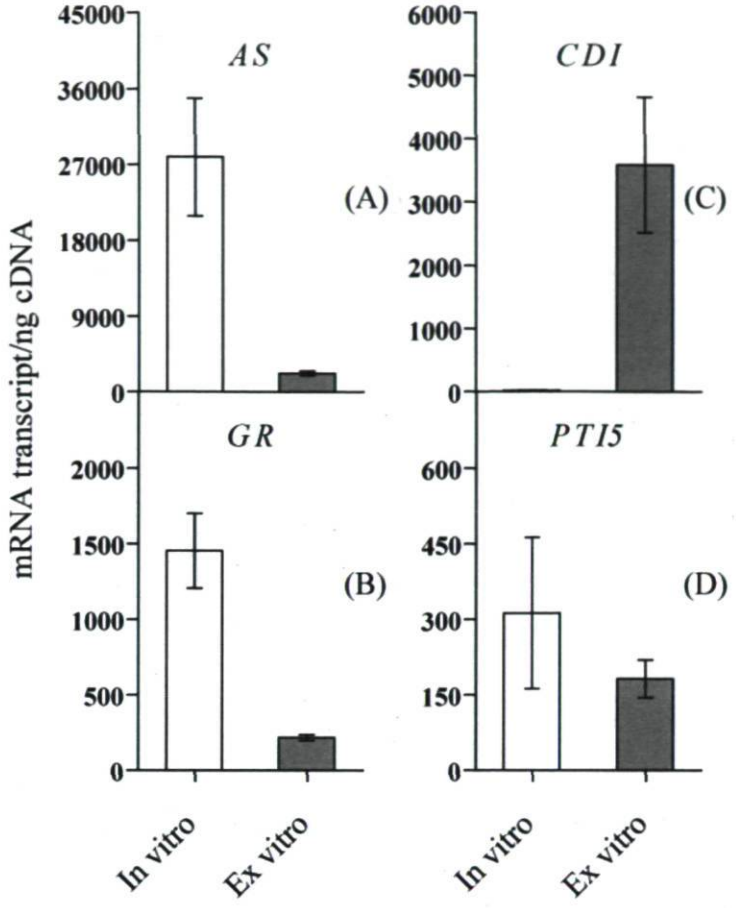

Figure 2.2 Validation of microarray expression data by real-time (RT) PCR analysis. Transcripts level of (A) asparagine synthetase (AS, AY240926), (B) glutathione reductase (GR, AW034391), (C) cathepsin D inhibitor (CDI, X73986) and (D) DNA-binding protein Pti5 (PTI5, U89256) were obtained by RT-PCR and their pattern compared with microarrays data. Total RNA was extracted from plantlets cultured *in vitro* for a period of 20 d (*in vitro*) and compared with plantlets cultured 20 d *in vitro* and transferred *ex vitro* for 12 d (*ex vitro*). AS, GR, CDI and PTI5 were respectively tagged as up-regulated, slightly up-regulated, down-regulated, and unregulated by microarrays analysis and RT-PCR in plantlets cultured *in vitro*. Data represent mean expression of five measurements ± SE. Expression data were normalized on the basis of cDNA amount as described by Libus and Storchova (2006). Result for AS, GR and CDI were statistically significant at $p \leq 0.05$.

2.6 Discussion

Here we report a first microarray experiment investigating the impact of *in vitro* culture conditions on plantlets. Data presented allows a better understanding of the perturbation of sink/source relationships, photosynthesis and nitrogen metabolism *in vitro*.

Furthermore, results indicated a strong response of defense-related genes and allowed the characterization of the stress response induced by the *in vitro* environment.

2.6.1 Modification of sink-source relationships in tomato plantlets grown *in vitro*

Carbohydrate and energy metabolism are key determinants for growth and development of plantlets, both *in vitro* and *ex vitro*. More precisely, plants synthesize and translocate sucrose from source to sink tissues in part by *SPS* (EC 2.4.1.14), which uses the triose phosphates produced during photosynthesis as substrates, and sucrose transporters (SUT), respectively. The expression data report that *SPS*, SUT and *LIN5* were down-regulated *in vitro*. LIN5, involved in phloem unloading in sink tissues (Roitsch & Gonzalez, 2004) and SUT4, have both been identified to be key determinant of sink strength (Weise *et al.*, 2000). Immunolocalization studies have indicated that *SUT4* is mainly expressed in sink leaves, stems and cotyledons and has been postulated to facilitate sucrose uptake in sink cells (Weise *et al.*, 2000). Additionally, the accumulation of sucrose in leaves was reported to inhibit transport activity of SUT4 (Chiou & Bush, 1998). The observed down-regulation of *LIN5*, *SUT4* and *SPS* expression suggests that the presence of exogenous sucrose *in vitro* causes feedback inhibition of plantlets sink leaf strength, and supports current hypotheses on the alteration of source/sink relationships *in vitro*.

Defective guard cells and open stomata are some of the important anatomical features for *in vitro* derived plant that would be responsible for uncontrolled water loss following transfer *ex vitro* (Brainerd & Fuchigami, 1982). Stomatal control is highly dependent on sucrose concentration in guard cell. In guard cells, AgpL1 is suggested to convert part of the sugars to starch at night, which will be hydrolysed and used as a source of malate, a counter ion to K^+, upon illumination (Xing *et al.*, 2005). The down-regulation of *AGPL1* expression (Park & Chung, 1998) *in vitro* could be a cause of the lack of stomatal responsiveness and high stomatal conductivity in tissue culture (Table 2.2). Hence, the impact of reduce level of AgpL1 expression on guard cells function *in vitro* must be investigated.

The analysis also revealed the lower expression of two genes involved in cellular respiration *in vitro* i.e. *HXK1* and ATPase β (Table 2.2). Expression of tomato *HXK1*::GFP

fusion protein indicated that *HXK1* is a type B hexokinase, associated with mitochondria, that is likely involved in sugar signaling (Damari-Weissler *et al.*, 2006). Over-expression of *HXK1* in *Arabidopsis* resulted in a hypersensitive response to glucose and to an altered growth and development pattern (Jang *et al.*, 1997). Regarding ATPase β, there is virtually no information available in the literature on its gene regulation, but our observations suggest that the abundance of sucrose in the medium could be responsible for the feedback inhibition of ATP synthesis in the respiratory chain. This hypothesis is further supported by the increased expression of Mn*SOD* in mitochondria *in vitro* (Fig. 2.1C), which is responsible for scavenging superoxides produced by respiratory complexes I and III in situation of high mitochondrial membrane potential or overreduced ubiquinone pool. These data suggest that the abundance of sucrose *in vitro* may lead to the inhibition of the expression of genes involved in the processing of endogenous sugars and the respiratory metabolism likely through the engorgement of sink roots by sugar

2.6.2 Low light and/or exogenous sucrose increase photosynthetic gene expression

It was found that different photosynthetic genes were up-regulated in plantlets cultured *in vitro*. Photosystems I and II in thylakoids are membrane protein complexes made of several different subunits. In the experiment, mRNA transcripts for the photosystem I K (*PSAK*) and photosystem II R (*PSBR*) were up-regulated *in vitro*. An analysis of *Arabidopsis* antisense transformants deficient in *PSAK* expression indicated that this protein participates in the interaction between the light harvesting complex (LHC) and the core antenna of photosystem I (PSI), and is essential to ensure stable assembly of the complex (Jensen *et al.*, 2000). It has been reported that PSAK could be of prime importance under low light conditions in order to provide a maximum efficiency and organization of PSI (Jensen *et al.*, 2000). This observation is supported by studies on *PSBR*, which also demonstrated that light is an important signal in the regulation of *PSBR* transcript while its expression was enhanced under low light conditions (Suorsa *et al.*, 2006). The increase transcript level of one subunit of both PSI and PSII could be attributed to the low light level *in vitro* (approx. 60 μmol photons m^{-2} s^{-1}). mRNA transcripts for *RCA* were also dramatically increased *in vitro* (Table 2.3). Past studies have indicated that the *in vitro* culture environment, and more precisely the presence of exogenous sucrose and low

CO_2, results in the reduction of net photosynthesis and inactivation of Rubisco and a reduction of catalytic site turnover rates of the enzyme (Desjardins *et al.*, 1993). In micropropagated plantlet, low sugar phosphate utilization due to sink limitation was shown to lead to end product inhibition of Rubisco activity. Hence, the high expression level of *RCA* may be required to release these tight binding inhibitors on Rubisco and suggests that sugar phosphate export is one of the limiting steps to carbon fixation *in vitro* due to high sugar concentration in the plant. These results are well supported by the inhibition of the chloroplast targeted NADP-dependent malic enzyme (NADP-ME) gene, *ME1*, (Table 2.2). As opposed to photosynthetic NADP-ME in C_4 plants, that concentrates CO_2 to Rubisco, studies on NADP-ME targeted to the chloroplast of C_3 plants have focused mainly on their involvement in the malate/oxaloacetate (OAA) shuttle. Chloroplast NAPD-ME use OAA and excess NADPH to produce malate. Afterward, malate can be exported to the mitochondria for ATP production, to the vacuole for storage or to the cytosol for the production of NADH for nitrate assimilation (Scheibe, 2004). According to current models, the shuttle would allow to store excess reducing power produced by photosynthesis, and to balance the ATP/NADPH ratio in the chloroplast. The lower photosynthetic rate of *in vitro* plantlets could explain the down-regulation of *ME1* mRNA. The impact of such a reduced expression level for *ME1* could be broad. In wheat, for example, a NADP-ME has been shown to ensure the production of NADPH for lignin biosynthesis and to be involved in wound response (Tausta *et al.*, 2002). These results indicate that low light and high sucrose content *in vitro* are important modulators of the photosynthetic genes expression and cell homeostasis, but the precise regulation cascade underlying this remains speculative.

2.6.3 High nitrate and ammonium up-regulate N assimilation *in vitro*

Carbon and nitrogen availability are important signals that interacts to adjust plant growth and development in response to nutrient status. The microarray results clearly indicated that *in vitro* culture conditions were inducing a specific expression pattern for genes linked to nitrogen metabolism. The expression of *NIR*, *GS* and *GOGAT* genes were significantly induced *in vitro* (Table 2.3), presumably associated with the abundance of sucrose and nitrogen in the Murashige and Skoog (MS) medium stimulating nitrate assimilation. The results point notably to the expression pattern of chloroplastic *GS2*, which is known to be up-regulated by light, sucrose, ammonium, and nitrate (Cren & Hirel, 1999).

In tomato, the source to sink transport of nitrogen is mainly achieved by glutamine and glutamate (Valle *et al.*, 1998). However, under a high nitrogen regime or stress conditions (Olea *et al.*, 2004), there is a significant increase of asparagine synthesis as a nitrogen rich compound in order to allow for higher nitrogen transport and storage. The up-regulation of *AS in vitro* is contrary to the down-regulation observed in tomato subjected to prolonged darkness (Devaux *et al.*, 2003). However, the up-regulation of *AS in vitro* is in accordance with Morcuende *et al.* (1998), who reported a synergistic effect of nitrate and sucrose feeding on asparagine accumulation in detached tobacco leaves. Similar results were also reported in *Arabidopsis* seedlings where exogenous amino acid restored the sucrose mediated repression of AS1 (Lam *et al.*, 1994). In *Oryza sativa* seedlings, asparagine synthesis has also been postulated to be involved in an ammonium assimilation/detoxification mechanism when ammonium concentration is high (Lam *et al.*, 1998). These results indicate that 20 days of growth under high nitrogen and sucrose conditions induced specific responses leading to processing and storage of excess nitrogen by *AS*. The data also indicate that transcripts for *NRT1.1* were down-regulated *in vitro* (Table 2.2). Contrary to previous studies showing low or no expression in tomato leaves (Lauter *et al.*, 1996), the *NRT1.1* transcript level was well over background values in our cDNA array experiment. The expression of NRT1 family gene in leaves has been observed in other species such as *Arabidopsis thaliana* and *Nicotiana plumbaginifolia* (Fraisier *et al.*, 2001). Recently, a role in nitrate sensing has been assigned to AtNRT1.1 (Remans *et al.*, 2006), and a similar role may be considered for tomato NRT1.1. The reduction in nitrate content experienced by plantlets upon transfer to *ex vitro* conditions could further increase the need to induce the nitrate sensing machinery. Hence, the reduction in *NRT1.1* mRNAs could also serve to cope with the excessive amount of nitrogen in the MS medium. The inhibition of nitrate transporters could limit the uptake of nitrate and ammonium to regulate nitrate assimilation with plantlets consumptions and needs. In this regard, the very high level of nitrate and ammonium in MS medium and the addition of sucrose could be responsible for this particular metabolic shift in nitrogen metabolism. The apparent necessity for storing the excess nitrogen may be related to sink limitation *in vitro* and could advantageously be used for growth upon transfer *ex vitro*.

2.6.4 *In vitro* plantlets are subject to a unique stressful environment

One of the distinctive characteristics of the *in vitro* environment is asepsis. It is normally assumed that the absence of pathogens and pests *in vitro* obviates the need for *in vitro* plantlets to activate biotic defense responses. Yet, defense mechanisms in plants can be induced by a plethora of environmental and biotic factors. An interesting finding of the present expression analysis is the large number of stress-related genes modulated by *in vitro* growth conditions. In this respect, we observed the up-regulation of the PR proteins *P4*, *P6*, *PR1a2* and *P23*. The P4 and P6 proteins are members of the PR-1 protein family and has been postulated to be involved in increasing cell wall strength and limit pathogen infection in the apoplast (Benhamou *et al.*, 1991; Santen *et al.*, 2005), even though their exact function is not yet known (van Loon & van Strien, 1999). The increase expression of *P4* and *P6* was unexpected as uninoculated tomato plants in greenhouse conditions usually do not present detectable levels of both mRNAs (Fidantsef *et al.*, 1999). Notably, it is worth mentioning that the high degree of homology of *P4* and *P6* could be compromising the capacity to distinguish both mRNA through microarray hybridization. Both PR-1 genes are induced by SA and are often considered as molecular markers of the systemic acquired resistance. Studies with PR1a2:: GUS fusion indicated that *PR1a2* expression is not induced by pathogen signals, SA and ethylene. This PR protein is presumably developmentally regulated (Tornero *et al.*, 1997). The results indicated that *PR1a2* was abundantly expressed *ex vitro* but that level of expression is further enhanced *in vitro*. Unlike its counterparts, P23 is a PR-protein member of the thaumatin-like family, named PR-5. Because of their homology to salt-stress proteins, the proteins of this family are also termed osmotins. In addition to their anti-fungal activity, the osmotins possibly play a role in the regulation of osmotic pressure, in the context of stress events like salinity, drought and cold. *P23* mRNA and protein was shown to be induced by ethylene, SA and various pathogens (Rodrigo *et al.*, 1993; van Loon *et al.*, 2006). Hence, the expression patterns revealed that several genes linked to the salicylic acid (SA) defense pathway were up-regulated *in vitro* 20 d following subculture. The increased expression of these stress-related genes suggests that the SA signaling cascade was induced *in vitro* and show evidence of the induction of the stress response in plantlets.

ROS are important regulators of gene expression and are involved in both biotic and abiotic stress responses. *In vitro* culture conditions differentially modulated the expression of ROS scavenging enzymes in mitochondria, chloroplasts and the cytosol. Past studies on mitochondrial MnSOD in tomato and *Trifolium repens* have demonstrated that this protein is induced when plantlets are grown in tissue culture conditions, and more specifically upon exposure to sucrose (Bowler *et al.*, 1989; Slesak *et al.*, 2006). The data are in accordance with these results, and the up-regulation of Mn*SOD* in presence of exogenous sucrose indicates a higher electron flow through the respiratory chain and an increased need to alleviate the oxidative stress in mitochondria. The increased production of ROS in the mitochondrial compartment could be a trigger for the induction of stress responses in *in vitro* plantlets (Bechtold *et al.*, 2005). It can also be postulated that heterotrophic growth increase the electron flow through the mitochondrial electron transport chain (mtETC), and the lower sink capacity of *in vitro* plantlets cause an excess electron pressure leading to higher superoxide production (Rhoads *et al.*, 2006). In the chloroplast, Cu-ZnSOD_{chl} gene have been found to be induced by ROS produced by photosynthesis and by different stress conditions (Kardish *et al.*, 1994). *In vitro* plantlets exhibited a lower Cu-ZnSODchl expression and therefore suggest that they present a decreased need for ROS scavenging in the chloroplast. This result indicate that even under low Rubisco activity, low light *in vitro* would lead to low excess excitation energy and consequently, lower ROS production. Inversely, the putative *APX* (*TL29*) gene was up-regulated *in vitro* (Table 2.3). Localization studies have shown *TL29* to be targeted to the thylakoïd lumen, presumably to process H_2O_2 produced by the photosystem II (Kieselbach *et al.*, 2000). This protein may also be involved in $^1O_2^*$ detoxification on the e^- donor side of PSII at the level of the Hill reaction, and/or in situation of overreduced pheophytin, Q_A and Q_B (Asada, 2006). Clearly, the *in vitro* environment produces a specific cell compartment pattern of ROS generation and scavenging that may lead to the observed defense responses. Therefore, the induction of SA signaling *in planta* may be associated with the cell redox status (Mateo *et al.*, 2006), which is highly dependent on sugars, light and photosynthesis.

In vitro plantlet display unique anatomical feature such as poorly differentiated leaf mesophyll and lower cuticle deposition. Ultimately, micropropagated plantlet can evolve toward the hyperhydric state where plant tissue exhibit lower lignin, cellulose and

differentiation (Kevers *et al.*, 2004). H_2O_2 and peroxidases are crucial determinants of cell growth, cell wall loosening, lignification and suberization, and lower H_2O_2 could trigger important alterations in *in vitro* plantlets leading to the *in vitro* phenotype. Modification in the spatial production and scavenging of ROS in leaf cells could explain certain characteristics of the *in vitro* plantlet phenotype. This hypothesis is further supported by the lower expression of Cu-ZnSOD_{cyt} (Fig. 2.1B) and *EXP18* in plantlets cultured *in vitro* (Table 2.2). The Cu-ZnSOD_{cyt} has been reported to accumulate in the apoplast, where it would be involved in the production of H_2O_2, an essential substrate for peroxidase involved in lignin biosynthesis (Ogawa *et al.*, 1996). Expansins, as primary cell wall loosening agents, play a critical role in plant cell expansion. These results suggest that aberrant growth and development of *in vitro* plantlets could be at least partially explained by ROS imbalance. This is in line with results discussed by Cassells et al. (2001) identifying oxidative stress as the main cause for the physiological, morphological and genetic defects observed in plants cultured *in vitro*. Additionally, we report that *in vitro* tomato plantlets exhibit an decrease expression of a LOX enzyme which are known to be involved in plant growth and development as a consequence of their function in membrane lipid oxidation. Accordingly, a close association was found between the rate of elongation and LOX enzyme activity (Siedow, 1991). The lower expression of these three enzymes involved in cell wall metabolism shed some light on the aberrant physiology observed *in vitro* and suggests that cell expansion may be affected by culture conditions.

As opposed to the SA defense signaling pathway, genes associated with the jasmonate signaling pathway were down-regulated in plantlets grown *in vitro*. The microarray analysis revealed that the *in vitro* culture conditions caused a lower expression of both *LOXC* and *AOS* (Table 2.2). The role of LOXs in plant defense resides mainly in their involvement in the conversion of linolenic acid to JA. Studies on the expression of *LOXC* have demonstrated that it is absent in leaves and not induced by wounding (Heitz *et al.*, 1997), which is surprising considering our results and considering the fact that the *LOXC* sequence shows 95% similarity with *Solanum tuberosum LOX2* which is activated in leaves upon wounding (Royo *et al.*, 1996). As for LOX, AOS is a key enzyme of the octadecanoid pathway involved in plant defense signaling. *AOS* transcripts are known to accumulate upon wounding and herbivore attacks (Howe *et al.*, 2000). The results suggest

that the octadecanoid pathway was repressed and synthesis of the signaling molecule jasmonate may be reduced *in vitro*. Upon wounding, jasmonate induces the synthesis of a wide diversity of compounds, including proteases and proteinase inhibitors. This inhibition was also partially reflected by lower mRNA levels observed for *CDI*, *SBT1* and *LAP-A* genes (Table 2.2). CDI was identified as a wound- and JA-inducible protein (Werner *et al.*, 1993), presumably involved in the inhibition of herbivores insects digestive proteases (Brunelle *et al.*, 2005). LAP-A, an enzyme involved in the hydrolysis of amino acids from the N-terminal extension of peptides and proteins (Gu *et al.*, 1996), has been postulated to be involved in protein turnover in tomato, helping the plant to recycle carbon and nitrogen in dying cell following pathogen attack (Gu *et al.*, 1999). Characterization of LAP-A in tomato demonstrated that it is stimulated when exposed to environmental challenges such as water deficit, salinity and abscisic acid, and also induced upon wounding and herbivory (Gu *et al.*, 1996). The subtilisin-like serine protease SBT1 has been characterized (Janzik *et al.*, 2000) and shown to be localized and active in the apoplast. Although the functions of SBT1 remain unknown, members of the subtilisin-like P69 serine protease family in tomato have been related to plant defense and postulated either to degrade pathogen proteins, or to be involved in the post-translational modification of defense proteins (Tornero *et al.*, 1996). These results suggest that the JA signaling pathway is impaired in *in vitro* plants, or stimulated after *ex vitro* transfer. Several studies have indicated that SA is a negative regulator of the JA-induced wound response (Wasternack *et al.*, 2006) and such interaction would explain the observed down-regulation of these stress-related genes. Recently, glutathione, a major cellular antioxidant, has been identified to be an important repressor of JA-responsive gene upon SA induction (Koornneef *et al.*, 2008). The up-regulation of *GR* expression, involved in the production of reduced glutathione, goes in line with Koornneef *et al.* (2008) observation and suggests that the down-regulation of JA-dependent gene expression is associated with SA-mediated redox regulation. However, while it is possible that JA signaling pathway is inhibited *in vitro* by the activation of the SA defense pathway, it can also be postulated that the acclimatization process could induce a stress cascade causing a response similar to wounding.

Tomato plantlets cultured for 20 d *in vitro* showed gene expression levels suggesting feedback inhibition on sink leaves strength, altered photosynthetic efficiency

and elevated nitrate assimilation. In particular, the presence of sucrose in the culture medium appears as a key determinant for many metabolic changes in *in vitro* plantlets and could be responsible for the specific cell ROS patterns, defense-related genes up-regulation and abnormal physiology. The *in vitro* environment induces a global genetic response far more complex than each component analysed on their own. Hence, the measurement of the sole impact of sucrose retrieval upon acclimatization *ex vitro* at the whole plantlet level will help to qualify the importance of heterotrophic growth on plantlets survival. In the light of the data presented here, *in vitro* plantlet would benefit from the implementation of a transitory phase *in vitro* which would consist in a reduction of sucrose concentration and an increase in light intensity. An intermediary acclimatization step would potentially favour plantlet survival *ex vitro* through improved photosynthetic capacity, reestablishment of normal sink/source relationship and alleviated oxidative stress.

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Chapitre 3 : Expression profiling of *in vitro* cultured tomato (*Solanum lycopersicum*) plantlets under sugar deprivation reveals important perturbation of ethylene signaling, cell wall metabolism and defense responses

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3.1 Résumé

Graduellement, au cours de leur développement, les feuilles vont passer d'un statut d'organe-puits, en tant qu'importateur net de carbone, à un statut d'organe-source, en tant qu'exportateur net de carbone. Lorsque transféré *ex vitro*, contrairement à cette transition progressive, les plantules cultivées *in vitro* mises en croissance en présence de sucre exogène doivent précipitamment atteindre l'autotrophie, plus particulièrement, en favorisant la photosynthèse. Les conditions *in vitro* entraînent chez les plantules d'importantes aberrations anatomiques et physiologiques qui les rendent inadaptés à l'environnement *ex vitro*. Pour ces raisons, l'étape de transfert vers le milieu *ex vitro* occasionne un important problème de survie chez les plantules. Pour étudier la réponse à court terme du retrait soudain du sucre exogène observé lors de l'acclimatation, des plantules de tomate (*Solanum lycopersicum*) *in vitro* ont été transférées dans un milieu de culture sans saccharose pendant 24 h. Le profile d'expression génique des plantules a été analysé à l'aide de la biopuce à ADN Tom2, présentant plus de 11 860 unigènes. Vingt-quatre heures après le retrait du saccharose, les plantules de tomate *in vitro* ne présentaient pas une réponse typique d'une carence en composé carboné. En revanche, le retrait du saccharose à plutôt causé un changement majeur dans l'expression de plusieurs gènes impliqués dans la modification de la paroi et de la membrane cellulaire, la biosynthèse et la voie de signalement de l'éthylène et les mécanismes de défense. Nous avons aussi remarqué que le retrait du saccharose modifie l'expression des gènes impliqués dans la déposition de la cire, la synthèse de lignine et les métabolismes des hormones. Ces résultats suggèrent que des changements métaboliques sont mis en œuvre rapidement après le retrait du saccharose et ceux-ci sont dictés par l'activation de la voie de signalement de l'éthylène. Cette réponse entraîne vraisemblablement la stimulation de processus physiologiques clés tels que l'élongation cellulaire, les mécanismes de défense et la sénescence et pourrait compromettre la survie des plantules lors de leur transfert *ex vitro*.

3.2 Abstract

During development, plant leaves gradually shift from being sink, as net importers, to becoming source, as a net carbon exporters. When transferred *ex vitro*, contrary to this ongoing transition, *in vitro* cultured plantlets grown in presence of exogenous sugar are forced to rapidly develop their autotrophic capabilities. Plantlets grown *in vitro* also develop an aberrant anatomy and physiology that is unsuitable to the *ex vitro* harsh environment. Therefore, the transfer of plantlets *ex vitro* often leads to important mortality. To study the short-term response of sugar deprivation observed following acclimatization *ex vitro*, *in vitro* tomato (*Solanum lycopersicum*) plantlets were transferred in sugar-free media for 24-h. The expression profile of these plantlets was analysed using the Tom2 microarray containing 11860 probes. After 24h of sugar deprivation, tomato plantlets do not show a typical famine response. Under these conditions, many regulated genes were involved in cell walls and membranes modification, ethylene biosynthesis and signaling, and defense response. We also observed that sugar deprivation interfered with the expression of genes involved in wax deposition, lignin synthesis and hormone metabolism. These results suggest that metabolic changes initiated shortly upon sugar deprivation are dictated by the activation of ethylene signaling pathway. This response might lead to the stimulation of important physiological processes such as cell elongation, defense responses and senescence all of which may compromise plantlets survival *ex vitro*.

Key words: *in vitro* culture, *ex vitro* culture, acclimatization, sucrose, *Solanum lycopersicum*, gene expression,

3.3 Introduction

Since its inception by Gottlieb Haberlandt in 1898, tissue culture has evolved into a high throughput technique for plant micropropagation. Despite the fact that tissue culture is utilized in various biotechnological methods, major bottlenecks are still encountered at the acclimatization stage, greatly reducing propagation efficiency. The major differences between the growth conditions *in vitro* and *ex vitro* have traditionally been held responsible for the important plantlet mortality. Indeed, contrarily to plants adapted to *in vivo* conditions, plantlets cultured *in vitro* are affected by high humidity, excessive nitrogen, limited CO_2 availability, low light and sucrose supplementation. These conditions are responsible for the development of unique anatomical and physiological abnormalities including malformed stomata, reduce cuticle thickness, poorly differentiated mesophyll layer, and poor photosynthetic capacity, to name just a few.

The first days following transfer *ex vitro* are especially important in determining the capacity of plantlets to survive as they are confronted with abrupt environmental changes. In particular, they must undergo important modification in leaf structure and photosynthesis during this acclimatization period. The newly transferred *ex vitro* plantlets must therefore produce new adapted leaf with increased cuticle and wax deposition, functional stomata and organized mesophyll tissues (Donnelly & Vidaver, 1984; Pospisilova *et al.*, 1999). Photosynthesis has been shown to be highly regulated by sucrose level *in vitro* (Kubota *et al.*, 2001; Le *et al.*, 2001; Yue *et al.*, 1993). High sucrose concentration was reported to negatively impact Rubisco and photosynthetic activity through increased cytosol hexose content and low inorganic phosphate concentration (Desjardins, 1995). Therefore, to survive during the early stages of acclimatization, it is assumed that plantlets must rapidly increase their photosynthetic activity to survive and resume growth under photoautotrophic conditions. However, the role of exogenous sucrose upon acclimatization is still nebulous. The retrieval or the reduction of sucrose from the *in vitro* media was shown to have a positive impact on growth and acclimatization of *Hypericum perforatum* (Couceiro *et al.*, 2006), *Rehmannia glutinosa* (Cui *et al.*, 2000), *Eucalyptus camaldulensis* (Kirdmanee *et al.*, 1995) and *Rosa* (Langford & Wainwright, 1987). By opposition, photomixotrophic culture condition was shown to have a positive effect on growth and acclimatization of

Alocasia amazonica (Jo *et al.*, 2009), *Persea americana* (Premkumar *et al.*, 2002) and *Cocoa nucifera* (Fuentes *et al.*, 2005). Moreover, growth, photosynthetic rate and internode length of *ex vitro Nicotiana tabacum* plantlet was increased in *in vitro* sugar-fed plantlets (Haisel *et al.*, 2001; Hofman *et al.*, 2002; Kadlecek *et al.*, 2001). The assessment of the effect of sugars is complicated by these conflicting results. On the one hand, exogenous sucrose seems to have a benefic impact on survival *ex vitro* possibly attributable to higher reserve and to their increase capacity to overcome *ex vitro* harsh conditions. On the other hand, exogenous sucrose would negatively impact plantlet's survival by slowing down transition toward autotrophy.

Among all the stress encountered by plantlets upon transfer *ex vitro*, sudden retrieval of sucrose from the culture media is surely the most important. Under normal conditions, the production of sugars through photosynthesis provides energy for growth but also constitutes an important environmental cue steering development. In *Arabidopsis thaliana* and *Oryza sativa* cell suspensions, sucrose starvation resulted in the up-regulation of mRNA transcripts coding for proteins involved in transcription, signal transduction, lipid breakdown, autophagy, senescence, and stress response and resulted in down-regulation of mRNA transcripts coding for enzymes involved in protein synthesis, glycolysis and cell division (Contento *et al.*, 2004; Wang *et al.*, 2007). While hexokinase is presumed to sense the status of cellular carbon (Xiao *et al.*, 2000), other studies attribute a role to trehalose as a metabolic switch allowing the fine control of sugar use and faith, in particular, by regulating sugar storage or utilization through starch metabolism (Wingler *et al.*, 2000). The fine control of these metabolic control points is essential to plantlets survival *ex vitro* during a period where sugar availability is transiently reduced.

Plants are highly plastic organisms and they are able to use fine tuning mechanisms to adapt to their environment. One of these mechanisms is the production of reactive oxygen species (ROS). Typically, change in environmental conditions result in the accumulation of cellular ROS which consequently initiates signaling cascades leading to change in gene expression. ROS are by-product of several metabolic pathway e.g. respiration, photosynthesis and fatty acid oxidation. In that manner, sugar is known to fulfill key functions in ROS production and scavenging (Couée *et al.*, 2006). Firstly,

soluble sugars play a pivotal role in the regulation of primary metabolic activities and antagonistically, in anti-oxidant process such as the oxidative pentose-phosphate pathway. The presence of exogenous sucrose *in vitro* has been recently shown to induce a specific cell ROS pattern with enhanced mitochondria SOD and reduced chloroplast and cytosol SOD gene expression (Dubuc, 2010; Slesak *et al.*, 2006). ROS metabolism governs several metabolic events such as synthesis of lignin, stomatal closure, stress and defense responses, cell division, and programmed cell death (Apel & Hirt, 2004; Slesak *et al.*, 2007).

Attempts to understand the aberration induce by the tissue culture process has relied until recently on biochemical approaches. Interrogating the transcriptome through large scale gene expression analysis now offers the possibility to deepen our understanding of this complex conundrum and better characterize the response of whole plantlets following *ex vitro* transfer. Earlier reports on the impact of *in vitro* culture conditions on plantlets metabolism using gene expression analysis showed that there is a extensive alteration in mRNA transcript associated with nitrogen assimilation, sink strength, and oxidative stress (Dubuc, 2010). It was also postulated that sugar constitute the most crucial component of the *in vitro* environment and possibly a determinant of the induction of defense-related genes and abnormal physiology of *in vitro* grown plantlets. In this respect, the objective of the present project was to measure the impact of short-term sugar deprivation, typically observed during the first few hours following transfer to acclimatization, imposed to plantlets transferred from *in vitro* to *ex vitro* culture conditions using microarray gene expression analysis. For this purpose, we subjected *in vitro* tomato plantlets to sugar deprivation for 24-h in order to better understand the impact of sucrose retrieval right after transfer to *ex vitro* acclimatization.

3.4 Materials and methods

3.4.1 Plant material and growth conditions

Solanum lycopersicum plantlets were micropropagated by internode cuttings at monthly intervals on MS medium supplemented with 8% bacto-agar (BD Diagnostic System, Sparks, MD, USA), and 3% sucrose. For the experiment, axillary apices were implanted on Sorbarod cellulose plugs (BaumGartner Paper, Lausanne, Switzerland) and

each placed solely in double stack magenta vessels (Sigma, Oakville, ON, Canada) containing 50 ml MS liquid medium with 3% sucrose. Apex were grown in a growth chamber (Conviron, Winnipeg, MB, Canada) under 180 µmol photons m^{-2} s^{-1}, 16-h photoperiod and 23/20°C day/night temperature. After a 20 day period, plantlets were transferred to fresh liquid medium containing either 3% (sugar feed) or no sucrose for 24-h (sugar deprived). The second leaf from each plantlet was harvest, and immediately frozen in liquid nitrogen and stored at -80°C.

3.4.2 RNA Extraction, cDNA synthesis, probe labelling and hybridization

The TOM2 array (Center for Gene Expression Profiling, Boyce Thomson Institute, Ithaca, New York), representing 11860 unigenes from *Solanum lycopersicum*, was used to conduct the gene expression analysis. The array was built using 69 nucleotides sequences from the Sol Genome Network (SGN) unigene database deposited on a Corning UltraGAPS™ coated slides (Corning, Lowell, MA). A total of six microarrays were used for this experiment. More precisely, three biological replicates represented by individually grown *in vitro* plantlet from both treatments were hybridized. Dye swap was conducted as technical replicate in order to minimize dye bias. Tissue grinding, total RNA isolation and cDNA synthesis were executed as described in chapter two of this thesis. Briefly, the Plant RNA Reagent (Invitrogen, Burlington, ON, Canada) was employed to extract total RNA. Quality was confirmed by Agilent 2100 Bioanalyzer (Agilent Biotechnologies, Missisauga, ON, Canada) microfluidic-based platform. Reverse transcription was realized using 20 µg total RNA. Aminoallyl-dUTP (Applied Biosystems, Streetsville, ON, Canada) was added to the reaction to enable later indirect labelling. Probe labelling was performed using AlexaFluor 555 or 647 reactive dye packs (Invitrogen) according to the manufacturer. An equal amount of each probe, ranging between 600 and 800 ng, was pooled and the SlideHyb buffer #1 (Applied Biosystems) added to the solution. TOM2 arrays were UV treated at 125 Mj (UVC500, Hoefer, San Francisco, CA) just prior hybridization. The hybridization was carried in a SlideBooster (Advalytix AG, Brunnthal, Germany)) instrument for 17-h at 42°C. Arrays were washed twice in both low and high stringency buffers as suggested by the SlideHyb buffer #1 protocol (Applied Biosystems).

3.4.3 Data analysis and Mapman visualization

Scanning, data acquisition, normalization and statistical analysis were performed as described in chapter two of this thesis. Briefly, arrays were visualized using the VersArray ChipReader (BioRad, Mississauga, ON, Canada) and the ChipReader 3.1 software (Bio-Rad) at 5-µm resolution. The microarray digital images were transferred to the Array-Pro Analyzer v.4.5 software (Media Cybernetics, Silver Spring, MD, USA) for spot detection and net intensity calculation. The background was estimated using the local ring method. The resulting spot intensities were log2 transformed, normalized using the global Loess method and statistically analysed using the Microarray Analysis of Variance (MAANOVA) software (Wu *et al.*, 2003), which operate under the R programming environment. P-values obtained were submitted to the false discovery rate (FDR) procedure. Genes harbouring an adjusted p-value lower than 0.01 and a fold change higher than 1.5 and lower than -1.5 were identified has been differentially regulated. The gene annotation used in this study was established by Blastx annotation of unigene sequences of tomato. Thus, we must be cautious in our interpretation as new published data could modify the sequence annotation. Microarray data were placed in the public repository "Gene Expression Omnibus (GEO)" (accession no. GSE14061, www.ncbi.nlm.nih.gov/geo) at NCBI.

3.4.4 MapMan/PageMan functional classification and analysis

Transcripts differentially regulated by sucrose treatment were assigned to their respective metabolic function using the MapMan and PageMan profiling tools (Thimm *et al.*, 2004; Usadel *et al.*, 2006). These softwares are useful tools to visualize data, to identify differentially regulated pathways and to detect metabolic trends. Fold changes for all unigene were $\log_2$ transformed and input into MapMan/Pageman. All unigenes present on TOM2 array were classified into 35 bins, representing functional categories. Wilcoxon rank sum test statistic was applied to data. The statistical analysis allows to test whether the median fold change for a specific bin differs from the median fold change of all genes in other functional classes.

3.4.5 Validation by real-time quantitative PCR

Real-time quantitative PCR (RT-PCR) was used to confirm results obtained with TOM2 oligo array. To proceed, primers were selected for fives genes identified as differentially regulated by sucrose treatment according to the microarray expression analysis. The Primer3Plus program was used to design PCR primers (Untergasser *et al.*, 2007). Primers and PCR products were submitted to the Basic local alignment search (BLAST) and strictly selected to minimize non-specific binding to another homolog tomato transcripts. The annotation and the sequence identifier of the Sol Genomic Network for the up-regulated genes selected for microarray validation are: SGN-U213523 (1-aminocyclopropane-1-carboxylate synthase (*ACC*)), SGN-U214425 (Ripening regulated protein DDTFR10/A (*DDTFR10/A*)), SGN-U216105 (Trehalose-6-phosphate phosphatase (*TPP*)) and SGN-U220883 (Xyloglucan endotransglycosylase 2 (*SlXTH2*)). In the same way, primers for the down-regulated mitochondrial small heat shock protein (*MT-SHSP*) (SGN-U212696) were designed. For cDNA synthesis, two µg of RNase free, DNase (Roche Diagnostics, Laval, QC, Canada) treated total RNA was reverse transcribed using Omniscript RT kit (Qiagen, Mississauga, ON, Canada) and oligo $d(T)_{18}$ (SUCOF, Quebec, QC, Canada) according to the manufacturer's protocol. Subsequently, the RNA of the hybrid cDNA-RNA was hydrolysed by the addition of 1 mM NaOH. Complementary DNA (cDNA) was quantified using the Quant-it RiboGreen RNA Reagent and Kit (Invitrogen) using the high range standard curve as described by Libus and Storchova (2006). Real-time PCR reactions were conducted using the Fast Start DNA SYBR® Green (Roche Diagnostic) with a Light Cycler™ Real-Time quantitative PCR instrument (Roche Diagnostic). Gene specific transcript of each sample was submitted to an absolute quantification using a standard curve, consisting of five dilutions of the original template. Amplification specificity was confirmed using a melting curve from 72°C to 95°C. Amplicons length and identity were validated by gel electrophoresis and DNA sequencing (CRCHUL, QC, Canada). Expression values were normalized on a basis of cDNA concentration and expressed as mRNA transcripts • ρg $cDNA^{-1}$. Statistical analysis was executed using the SAS software (SAS Institute, Cary, NC).

3.5 Results

For this study, tomato plantlets were grown for 20 days under *in vitro* culture conditions (3% sucrose) before being transferred to 0% sucrose or maintained in 3% sucrose for 24h under *in vitro* conditions. Using the oligo TOM2 array, representing 11860 unigenes, we have identified differentially-regulated genes expressed upon sugar retrieval. After a full day of sugar deprivation, a total of 251 genes were identified to have significantly different transcript levels (adjusted p-value ≤ 0.01). A $\pm$ 1.5-fold change cut-off was chosen in order to obtain a comprehensive list of responsive genes. Amongst the responding genes, 144 were induced and 107 were repressed. More precisely, 80, 37 and, 20 up-regulated genes and 53, 24 and, 9 down-regulated genes showed a 2-, 2.5- and, 3-fold changes of expression, respectively.

MapMan (Thimm *et al.*, 2004) and PageMan software (Usadel *et al.*, 2006) were used to identify groups of genes regulated in a synchronized manner. Thus, all genes on the TOM2 array were assigned to 35 bins, each divided into sub-bins, and submitted to Wilcoxon test (Table 3.1). This analysis revealed that genes involved in nucleotide recycling, protein degradation and post-translational modification were up-regulated, while those involved in protein synthesis and targetting were down-regulated. Sugar deprivation also had a striking effect on genes involved in the regulation of transcription. Genes associated to AP2/EREBP, WRKY and GeBP like transcription factor families were up-regulated while those associated to RNA processing and transcription were down-regulated in a coordinated manner. Genes involved in chromatin structure such as histones and SET-domain transcriptional regulators were down-regulated. Sucrose retrieval also decreased the expression of genes involved with mitochondrial electron transport, glycolysis, oxidative pentose phosphate pathway, and transport but increased those involved in gluconeogenese. Genes involved with brassinosteroid and cytokinin signal transduction were up-regulated whereas genes involved with abscisic acid

Table 3.1: Functional categories significantly altered under sugar deprivation *in vitro* identified by MapMan profiling tool.

Bin	Name	Elements	P-value[a]
Up-regulated[b]			
2.2.1.1	major CHO metabolism.degradation.sucrose.fructokinase	7	0.0180
5	fermentation	21	0.0180
5.1	fermentation.aldehyde dehydrogenase	16	0.0300
6.4	gluconeogenese/ glyoxylate cycle.PEPCK	3	0.0290
13.1.3.4.12	amino acid metabolism.synthesis.aspartate family.methionine.S-adenosylmethionine synthetase	3	0.0380
13.2.4.4	amino acid metabolism.degradation.branched-chain group.leucine	5	0.0050
13.99	amino acid metabolism.misc	2	0.0480
14.1	S-assimilation.APS	3	0.0230
17.3.2.99	hormone metabolism.brassinosteroid.signal transduction.other	3	0.0340
17.4.2	hormone metabolism.cytokinin.signal transduction	2	0.0440
23.3	nucleotide metabolism.salvage	10	0.0390
26.11	misc.alcohol dehydrogenases	18	0.0280
27.3	RNA.regulation of transcription	1156	0.0410
27.3.3	RNA.regulation of transcription.AP2/EREBP family	58	0.0003
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	50	0.0120
27.3.49	RNA.regulation of transcription.GeBP like	5	0.0180
27.3.62	RNA.regulation of transcription.Nucleosome/chromatin assembly factor group	6	0.0495
29.4	protein.postranslational modification	384	0.0002
29.5.4	protein.degradation.aspartate protease	21	0.0310
29.5.11.4	protein.degradation.ubiquitin.E3	266	0.0110
29.5.11.4.2	protein.degradation.ubiquitin.E3.RING	160	0.0073
30.1	signalling.in sugar and nutrient physiology	17	0.0170
Down-regulated			
2.2.2	major CHO metabolism.degradation.starch	31	0.0350
4.5	glycolysis.pyrophosphate-fructose-6-P phosphotransferase	5	0.0017
7	OPP	30	0.0280
7.3	OPP.electron transfer	5	0.0490
9.1	mitochondrial electron transport / ATP synthesis.NADH-DH	14	0.0200
9.1.2	mitochondrial electron transport / ATP synthesis.NADH-DH.localisation not clear	14	0.0200
10.1.4	cell wall.precursor synthesis.UGD	3	0.0470
13.1.2	amino acid metabolism.synthesis.glutamate family	6	0.0495
17.1	hormone metabolism.abscisic acid	28	0.0160
17.1.3	hormone metabolism.abscisic acid.induced-regulated-responsive-activated	9	0.0280
20.2.1	stress.abiotic.heat	104	0.0110
22.1.3	polyamine metabolism.synthesis.arginine decarboxylase	5	0.0130
23.4.3	nucleotide metabolism.phosphotransfer and pyrophosphatases.uridylate kinase	2	0.0320
26.1	misc.misc2	19	0.0140
26.9	misc.glutathione S transferases	40	0.0470
27.1	RNA.processing	122	0.0320
27.1.20	RNA.processing.degradation dicer	8	0.0200
27.2	RNA.transcription	54	0.0120
27.3.69	RNA.regulation of transcription.SET-domain transcriptional regulator family	24	0.0390
28.1.3	DNA.synthesis/chromatin structure.histone	51	0.0410
29.1	protein.aa activation	44	0.0280
29.1.20	protein.aa activation.phenylalanine-tRNA ligase	6	0.0320
29.2.2	protein.synthesis.misc ribososomal protein	209	0.0020
29.3	protein.targeting	129	0.0290
29.5.11.20	protein.degradation.ubiquitin.proteasom	33	0.0062
30.2.10	signalling.receptor kinases.leucine rich repeat X	7	0.0220
30.11.1	signalling.light.COP9 signalosome	8	0.0260
34	transport	439	0.0340

[a] Analysis was carried out using MapMan profiling tools. For each gene, the fold change between sugar deprived and sugar feed plantlet was calculated. Fold change were $\log_2$ transform and a Wilcoxon test was performed to identify significantly regulated functional class.

[b] Functional category were classified as whether their average signal increase (up-regulated) or decreased (down-regulated) in the respective bin, under sugar deprivation.

metabolism were down-regulated. Sucrose deprivation caused the coordinated down-regulation of leucine rich repeat X receptor kinase and light signaling (COP9 signalosome) and up-regulated sugar and nutrient signaling. Analysis also reported an important decrease of gene associated with heat stress and glutathione S-transferases functional categories. Somewhat unexpectedly, genes related to starch degradation were down-regulated.

3.5.1 Transcriptional regulation of cell wall metabolism in response to sugar deprivation

A considerable number of genes linked to cell wall metabolism were significantly regulated upon transfer from heterotrophic to autotrophic conditions (Table 3.2). Interestingly, five genes out of twenty showing a higher expression (3-fold higher mRNA transcript level) were related to cell wall cleavage, rearrangement and synthesis. In particular, we observed an increased expression for genes involved in nucleotide sugar synthesis, and cell wall degradation, rearrangement, synthesis and structure. Sugar deprivation initiated the transcriptional up-regulation of a pectinesterase and a β-galactosidase, involved in the breakdown of cell wall polysaccharide backbone. Moreover, a β-1,4-xylosidase was up-regulated 5.5-fold, one of the highest increase in expression observed in this experiment. Inversely, a xylosidase and a β-1,4-D-glucanase were repressed by 1.5- and 1.7-fold, respectively. A UDP-glucose-4-epimerase, involved in cell wall synthesis, was up-regulated 1.6-fold following sucrose retrieval. Similarly, two genes from the myo-inositol pathway, namely a myo-inositol 1-phosphate synthase and a myo-inositol oxygenase, also involved in cell wall polysaccharide biosynthesis, were up-regulated 2.8- and 3.7 fold. Additionally, two xyloglucan endotransglycosylases, *SlXTH2* and *SlXTH5* involved in cell wall modification, were also up-regulated 4.5- and 3.3-fold in sugar deprived plantlets. Gene expression of structural cell wall proteins, such as the extensin-like protein NTEIG-C29 and two proline-rich proteins were up-regulated after sugar deprivation.

Table 3.2: Expression of cell wall metabolism- and cell membrane-associated genes up- and down-regulated after 24h of sugar deprivation under *in vitro* culture conditions.

Functional classification		Gene annotation	SGN no.[a]	Fold induction
Cell wall	Cleavage and modification	Beta-1,4-xylosidase	U222710	5.5
		Xyloglucan endotransglycosylase SlXTH2	U220883	4.5
		Pectinesterase	U214451	3.7
		Xyloglucan endotransglucosylase-hydrolase SlXTH5	U213423	3.3
		Beta-galactosidase	U214121	2.0
		Xylosidase	U225400	-1.5
		Endo-beta-1,4-D-glucanase	U218121	-1.7
	Synthesis	Putative inositol polyphosphate-5-phosphatase At5P2	U215067	1.9
		Myo-inositol 1-phosphate synthase	U213443	2.8
		Myo-inositol oxygenase	U214186	3.7
		UDP-Glc-4-epimerase	U214352	1.6
		Myo-inositol-1-phosphate synthase	U213442	-1.8
	Structural component	Extensin-like protein NtEIG-C29	U213037	2.2
		Proline-rich protein-related	U217499	1.6
	Lignin and cuticle biosynthesis	Putative peroxidase	U213345	1.9
		Elicitor-activated gene (ELI3)	U219296	-2.1
		Eceriferum (CER1)	U215098	-2.1
		Lignin forming anionic peroxidase precursor	U215231	-2.5
	Lipid transfer protein	Nonspecific lipid-transfer protein 1 precursor (LTP 1)	U213641	2.2
		Protease inhibitor/seed storage/lipid transfer protein (LTP) family	U232551	2.1
		TSW12 (probable lipid transfer protein)	U232089	-2.2
		TSW12 (probable lipid transfer protein)	X56040[b]	-2.2
		Putative lipid transfer protein	U217389	-2.6
		Nonspecific Lipid Transfer Protein Complexed With Palmitate	U222218	-3.2
		Putative non-specific lipid transfer protein StnsLTP	U216420	-3.6
Cell membrane	Synthesis, cleavage and modification	Putative sphingolipid delta 4 desaturase DES-1	U213173	3.2
		Lipase-like protein	U242699	2.2
		Esterase/lipase/thioesterase family	U217460	2.0
		Lipoxygenase	U240112	2.0
		Microsomal omega-6-desaturase	U214192	1.9
		Lipid desaturase-like protein	U232815	1.6
		Fatty acid desaturase family protein	U217678	-4.1

[a] Unigene identifier from Sol genome network (SGN)
[b] Genbank accession number
[c] Statistical analysis was done by using the F-test of the R/maanova package (Wu *et al.*, 2002). Only genes showing an adjusted p-value under 0.01 and a fold change higher than 1.5 and lower than -1.5 are presented.

3.5.2 Effect of sucrose retrieval on lipid metabolism

The cell membrane plays a crucial role in many cellular processes like transport, signaling and cytoskeleton anchoring. The microarray data revealed that several enzymes involved in the architecture and signaling function of the cell membrane were modulated under sugar deprivation (Table 3.2). Four fatty acid desaturases were differentially regulated upon transfer from heterotrophic to autotrophic conditions. Desaturases are enzyme involved in the formation of a double bond by releasing two hydrogen atoms and therefore modify biophysical properties of the cell membrane phospholipids. In particular, a lipid desaturase-like protein, a microsomal omega-6-desaturase (*FAD2*) and a sphingolipid

delta-4 desaturase (*DES-1*), also called dihydroceramide desaturase, were up-regulated 1.6-, 1.9- and 3.2-fold under sugar deprivation. In contrast, another putative fatty acid desaturase was repressed 4-fold suggesting a coordinated regulation of lipid biosynthesis in plantlet cells. Polyunsaturated fatty acid (PUFA), such as 18:2 and 18:3 produced by desaturase, can be further oxidized by the enzyme lipoxygenase (LOX). A *LOX* gene was up-regulated 2-fold under sugar deprivation. Despite the induction of several mRNA transcripts involved in lipid biosynthesis, a lipase-like protein and an esterase/lipase/thioesterase family protein mRNA involved in lipid breakdown were reported to be up-regulated 2.2- and 2-fold under sugar deprivation.

In parallel, plants lay down a large amount of fatty acid into the cuticle as an additional barrier protecting plant from environmental stresses. The eceriferum (*CER1*) gene involved in wax biosynthesis was down-regulated 2.1-fold in sucrose deprived plantlets (Table 3.2). Additionally, we also observed the 1.9-fold down-regulation of a cuticular protein. The expression of two lipid transfer protein (LTP) was up-regulated while four others were down-regulated (Table 3.2). Notably, the mRNA level of *TSW12* and three other non-specific LTP (nsLTP) were repressed by 2.2-, 2.6-, 3.2- and 3.6-fold after 24h of sugar deprivation. Lipid transfer proteins are small (7-10kDa) protein generally targeted to the extracellular matrix that exhibit lipid-binding properties.

3.5.3 Alteration of secondary metabolism transcript levels

Our microarray result points to a specific regulation of secondary metabolism under sugar deprivation. Genes coding for key enzymes involved in lignin biosynthesis were repressed in this experiment. After 24-h of sugar deprivation, the elicitor-activated (*ELI3*) gene, which is reportedly coding for a cinnamyl alcohol dehydrogenase (*CAD*), showed lower expression (Table 3.2). Subsequently, monolignols are transported to the cell wall, where they will undergo dehydrogenative polymeration to form lignin. In this line of thought, the expression of a lignin forming anionic peroxidase was repressed 2.5-fold (Table 3.2). These results are also correlated with a major decreased in chorismate synthase 2 (*CS2*) mRNA level, an important enzyme from the shikimate acid pathway which leads to salicylic acid and lignin biosynthesis (Table 3.3).

Table 3.3: Expression of primary, amino acid and protein metabolism genes up- and down-regulated after 24h of sugar deprivation under *in vitro* culture conditions.

Functional classification	Gene annotation	SGN no. [a]	Fold induction
Photosynthesis	Ribulose bisphosphate carboxylase small chain 3A/3C, chloroplast precursor	U225541	2.6
	Carbonic anhydrase, chloroplast precursor	U213080	1.5
Carbohydrate metabolism	Trehalose phosphatase family	U216105	2.6
	Putative trehalose-6-phosphate synthase	U216992	1.8
	Starch synthase	U229655	-2.2
	Invertase - like protein	U214570	-2.8
Glycolysis	Phosphoenolpyruvate carboxylase	U219468	2.2
	Phosphoenolpyruvate carboxylase kinase	U213890	-2.0
Transporter	Putative sugar transporter	U231225	3.4
	Nitrate transporter NRT1-1	U229252	2.8
	Putative sugar transporter	U233855	1.9
	Putative monosaccharide transporter 3	U217741	1.8
	Phosphatidyl-inositol-transfer protein domain	U241951	1.8
	Putative ABC transporter transmembrane protein	U214603	-1.5
	Transporter-related	U216139	-1.5
	Putative phosphatidylinositol transfer	U226582	-1.5
	Putative zinc transporter	U219146	-1.7
	Chloroplast channel forming outer membrane protein	U215773	-1.7
	Amino acid transporter family	U222988	-1.8
	Putative sugar transport protein	U220395	-1.8
	ABC transporter family protein	U226307	-1.9
	Putative outward rectifying potassium channel (KCO5)	U229952	-2.1
	Nitrate transporter	U231924	-2.3
Respiration	Mitochondrial F1-ATPase epsilon subunit	U227392	1.8
	Ubiquinol-cytochrome C reductase Fe-S subunit 5 (cyt bc1)	U232104	1.7
	Frataxin protein-related	U221460	-1.6
	NADH:ubiquinone oxidoreductase (UQ)	U234069	-1.8
	Cytochrome c (cyt c)	U229359	-1.9
	Ubiquinol-cytochrome-c reductase (cyt bc1)	U212761	-2.2
Nitrogen and amino acid metabolism	Ornithine carbamoyltransferase	U234101	3.2
	Uridylyltransferase-related	U227541	2.2
	Beta-alanine-pyruvate aminotransferase	U215551	2.2
	Serine acetyltransferase	U214945	2.0
	Glutamate decarboxylase 3	U212559	1.8
	Nitrate reductase	X14060	1.7
	Cystathionine beta-lyase	U219944	1.5
	S-adenosylmethionine decarboxylase (AdoMetDC)	U213084	-1.5
	L-allo-threonine aldolase	U219021	-1.8
	Gamma hydroxybutyrate dehydrogenase	U214601	-2.2
	Putative branched chain alpha-keto acid dehydrogenase subunit E2	U221864	-2.7
	Chorismate synthase 2	U232478	-3.2
Protein synthesis and degradation	60S ribosomal protein L10A	U241724	3.5
	Kruppel-like protein	U217190	2.6
	E3 ubiquitin ligase (RMA1)	U217504	2.1
	Translation-inhibitor protein	U213124	1.9
	RING zinc finger protein -related	U221443	1.8
	E3 ubiquitin ligase SCF complex subunit, SKP1/ASK1	U215182	1.8
	Autophagy protein (APG8H)	U234765	1.8
	Eukaryotic release factor 1 homolog	U233748	1.6
	Aspartic proteinase 2	U217420	-1.7
	Putative RING zinc finger protein-like protein	U220801	-2.0
	50S ribosomal protein L29	U216312	-2.0
	Ribosomal protein L28-like	U213137	-2.0
	Putative RING-H2 finger protein (RHB1a)	U216889	-2.6

[a] Unigene identifier from Sol genome network (SGN)

[b] Genbank accession number

[c] Statistical analysis was done by using the F-test of the R/maanova package (Wu *et al.*, 2002). Only genes showing an adjusted p-value under 0.01 and a fold change higher than 1.5 and lower than -1.5 are presented.

3.5.4 Effect of sugar deprivation on primary, amino acid and protein metabolism

Somewhat unexpectedly, very few genes involved in plant carbon metabolism and photosynthesis were modulated by sugar deprivation (Table 3.3). For instance, a starch synthase and a cytoplasmic neutral invertase were down-regulated, while the expressions of one and three sugar transporters were respectively down- and up-regulated upon sucrose retrieval. The photosynthetic gene ribulose-1,5-bisphosphate carboxylase small subunit 3A/3C and carbonic anhydrase transcripts were up-regulated by sucrose deprivation. Furthermore, trehalose metabolism responded to sugar deprivation; both a trehalose-6-phosphate synthase (*TPS*) and a trehalose-6-phosphate phosphatase (*TPP*) genes were up-regulated.

Genes involved in the respiratory pathway were affected by sugar deprivation (Table 3.3). In particular, the expressions of ubiquinone (*UQ*), cytochrome bc1 (*CYT BC1*) and cytochrome C (*CYT C*) were repressed. UQ, CYT BC1 and CYT C are all involved in keys steps of the mitochondrial electron transport chain (ETC). A frataxin protein-related gene which is apparently involved in iron-sulphur cluster protein assembly and mitochondrial iron efflux was also down-regulated under sugar deprivation. Despite the repression of different ETC components, the epsilon subunit of mitochondrial F1-ATPase was up-regulated possibly suggesting an adaptation to lower respiratory activity.

Amino acid metabolism was particularly active following transfer to autotrophic conditions (Table 3.3). Genes of five enzymes involved in amino acid biosynthesis, namely glutamate decarboxylase (*GAD*), beta-alanine-pyruvate aminotransferase, ornithine carbamoyltransferase, cystathionine beta-lyase, serine acetyltransferase were up-regulated. In parallel, two proteins involved in amino acid degradation, the branched chain alpha-keto acid dehydrogenase and L-allo-threonine aldolase, were down-regulated. It is interesting to note that sugar deprivation positively affected the *GAD* and negatively regulated the gamma hydroxybutyrate dehydrogenase (*GHBDH*) transcripts level, both of which are involved in GABA metabolism. Many transcripts involved in protein synthesis and degradation were also differentially regulated (Table 3.3). The 60S ribosomal protein (*RPL10a*) and eukaryotic release factor 1 (*ERF1*) involved in translation were stimulated by

sugar deprivation. Despite this induction, two others ribosomal proteins, *L29* and *L28-like*, were repressed. Especially, we have identified four transcript members of the E3 ubiquitin ligase family that were induced and two inhibited. These enzymes participate in the final steps of ubiquination and interact with specific proteins for targeting towards the proteasome. Interestingly, the transcript level of an autophagy protein (*APG8H*) was slightly induced after plantlet transfer to autotrophic conditions.

Sudden changes in medium carbon availability cause important modifications in reallocation of resources in the entire plant. In this perspective, several transporters were differentially regulated upon sugar deprivation (Table 3.3) as some nitrate transporter and amino acid transporter were simultaneously up- and down-regulated. Our analysis also revealed the down-regulation of a zinc transporter and a calcium activated outward rectifying potassium channel (chloroplastic) in sugar deprived plantlets. We also noted the down-regulation of a chloroplast channel forming outer membrane protein. Moreover, two ABC transporters were down-regulated when plantlets were transferred to 0% sucrose.

3.5.5 Transcription factors, signaling and cell organisation

Transcriptional regulation of transcription factors (TF) can reveal important cues on the genetic responses of *in vitro* plantlets subjected to sugar deprivation. The microarray analysis revealed that many TF were regulated by sucrose retrieval (Table 3.4). Globally, a higher proportion of transcription factors and higher fold changes were reported for up-regulated compared to down-regulated TF. Accordingly, six out of twenty genes showing higher than 3-fold increase in expression under sugar deprivation were coding for transcription factors. In particular, families of up-regulated transcription factors following sucrose retrieval include AP2/EREBP, G2-like, TCP, WRKY, NAC and Aux/IAA (Table 3.4). Data analysis also revealed that CCR4-associated factor *(CAF1)*, involved in the regulation of cell wall protein gene expression (Sarowar *et al.*, 2007) was up-regulated 2.5-fold in expression when plantlets were transferred to 0% sucrose (Table 3.4). In opposition, down-regulated transcription factors include MADS box, MYB and bZip. Transcription factors families showing mitigated regulation include bHLH, C2H2(Zn) and C2C2(Zn)-gata.

Upon transfer from heterotrophic to autotrophic culture condition, phytochrome B1 (*PHYB1*) showed decrease transcript levels in leaves of tomato plantlets (Table 3.4). Additionally, transcripts of genes involved in calcium signaling such as a calmodulin, the calcium-dependent protein kinase 1 (*CDPK1*) and a calcium-binding protein were down-regulated, while the calcineurin B-like protein 1 (*CBL1*) was up-regulated in sugar deprived plantlet (Table 3.4).

Table 3.4: Expression of genes involved in transcription and signaling up- and down-regulated after 24h of sugar deprivation under *in vitro* culture conditions.

Functional classification	Gene annotation	SGN no. [a]	Fold induction [b]
Transcription	bHLH protein family	U227453	7.1
	Myb family transcription factor	U222766	6.2
	Putative auxin-induced protein, IAA17/AXR3-1	U218627	4.5
	AG-motif binding protein-1	U239025	3.2
	Putative CHP-rich zinc finger protein	U223063	3.1
	Transcriptional activator CBF1	U216204	3.0
	Zinc finger (CCCH-type) family protein	U220600	2.9
	CCR4-associated factor 1-related protein	U215603	2.5
	Ripening regulated protein DDTFR10/A	U214425	2.3
	NAC domain containing protein 2	U214595	2.1
	AP2 domain transcription factor	U228673	2.1
	Lsd1 like protein	U223548	2.0
	Transcriptional co-activator (KIWI) -related	U223109	2.0
	TCP-domain protein	U227424	2.0
	Putative zinc finger protein	U214710	1.9
	Transcriptional adaptor like protein	U216582	1.8
	WRKY family transcription factor	U226247	1.7
	MYB-like DNA-binding domain protein	U220637	-1.5
	Zinc finger (C2H2 type) family protein	U216762	-1.5
	CONSTANS B-box zinc finger family protein	U218448	-1.6
	RNA-directed RNA polymerase	U217771	-1.7
	bZIP transcription factor BZI-2	U219333	-2.4
	MADS-box transcription factor-like protein	U223527	-2.5
	Putative DHHC-type zinc finger domain-containing protein	U220992	-2.7
	Dof zinc finger protein	U221271	-2.9
Signaling	Calcineurin B-like protein 1 (CBL1)	U215621	2.6
	Serine/threonine-specific protein kinase NPK15	U222547	2.0
	Leucine rich repeat protein family	U220972	-1.6
	Calcium-dependent protein kinase	U212665	-1.7
	Calmodulin	U212856	-1.8
	Copia-type pol polyprotein-like	U216731	-2.2
	Probable calcium-binding protein	U213630	-2.2
	Calcium-dependent protein kinase CDPK1	U214963	-2.2
	Phytochrome B1	U229748	-2.5

[a] Unigene identifier from Sol genome network (SGN)

[b] Statistical analysis was done by using the F-test of the R/maanova package (Wu *et al.*, 2002). Only genes showing an adjusted p-value under 0.01 and a fold change higher than 1.5 and lower than -1.5 are presented.

Cell sugar status is a crucial determinant in the control of cell cycle (Gibson, 2004). Interestingly, some genes closely related to cell plate formation in cytokinesis were down-regulated under sugar deprivation. Accordingly, the microarray analysis revealed that the

plant 135kDa actin bundling protein (*P-135-ABP*) was down-regulated 3.7-fold after 24-h of sugar deprivation (data not shown).

3.5.6 Hormones

Plantlets transferred to sucrose free medium displayed considerable modification in mRNA transcripts related to hormone metabolism. When submitted to sugar deprivation, genes linked to ethylene synthesis, signaling, and responses were up-regulated (Table 3.5). This was clearly the case for the 1-aminocyclopropane-1-carboxylate synthase (*ACC*) which is a major enzyme responsible for ethylene biosynthesis. In addition, three transcription factors belonging to the Apetala2/Ethylene-responsive element binding protein family (AP2/EREBP) followed the same trend. Genes linked to the auxin response, more specifically two auxin-regulated stem proteins, one auxin-repressed protein, and the indole-3-acetic acid 17/Auxin Resistant 3 (*IAA17/AXR3*) were stimulated when sucrose was retrieved from the growth medium. By opposition, the expression of the gibberellin 20-oxidase-1 (*GA20OX1*) and geranyl diphosphate synthase (*GPS*) declined in sugar deprived plantlets (Table 3.5). The jasmonic acid biosynthetic gene lipoxygenase 3 (*LOX3*) and allene oxide cyclase (*AOC*) were respectively up- and down-regulated by sucrose deprivation (Table 3.5). The transcript level of a unigene homolog to the steroid sulfotransferase 4 from *Brassica napus,* which encodes an enzyme involved in brassinosteroids sulfonation (Marsolais *et al.*, 2004) was up-regulated in sugar deprived tomato plantlets (Table 3.5). Thus, transcripts associated to hormone metabolism were responsive to sucrose treatment and their differential regulation could underpin important modifications in plantlets growth and development.

3.5.7 Modulation of stress-related genes expression by sucrose

The induction of defense-related genes is a generalized response of plants when they are confronted to adverse biotic, abiotic stress and many environmental cues. Many transcripts regulated by sucrose level were associated with plant defense (Table 3.5). Notably, heat shock proteins (HSP) were very responsive to sugar deprivation. In our experiment, five members of the Hsps family were down-regulated while three were up-regulated by the removal of sugar. Indeed, two DnaJ domain-containing proteins and a

DnaJ-like protein were induced and a DnaJ homolog was repressed. By opposition, several small HSP (*sHSP*) were down-regulated, including a mitochondrial sHSP (*MT-SHSP*) and three cytosolic sHSP (*HSP17.6*, *HCT2*, *HSP20.0*). The sHSPs may facilitate protein folding by interacting and stabilizing non-native proteins to facilitate their refolding by other HSPs.

Table 3.5: Expression of genes involved in hormone metabolism and stress-related process up- and down-regulated after 24h of sugar deprivation under *in vitro* culture conditions.

Functional classification	Gene annotation	SGN no. [a]	Fold induction [b]
Hormone	1-aminocyclopropane-1-carboxylate synthase	U213523	2.4
	Auxin-repressed protein	U213615	2.2
	Steroid sulfotransferase 4	U217397	2.0
	Expressed protein stem-specific protein	U232815	1.6
	Expressed protein stem-specific protein	U216663	1.6
	Gibberellin 20-oxidase-1; 20ox-1	U226448	-1.5
	Geranyl diphosphate synthase	U224450	-1.8
	Allene oxide cyclase	U217432	-2.8
Stress	PR5-like protein	U213935	3.7
	Avr9/Cf-9 rapidly elicited protein 65	U225130	2.5
	Pathogenesis-related protein (PR-5 protein)	U213934	2.4
	Elicitor inducible protein	U216295	2.3
	Putative shock protein SRC2	U222500	2.2
	Pathogenesis-related leaf protein 6 precursor (P6)	U212922	2.1
	DnaJ-like protein	U216888	2.1
	DnaJ domain-containing protein	U223323	2.0
	Elicitor-inducible protein (EIG-J7)	U217308	1.7
	Proteinase inhibitor I	U213023	1.7
	Beta-1,3-glucanase-like protein	U220181	1.6
	Cold acclimation protein WCOR413-like protein	U214795	1.6
	Proteinase inhibitor II TR8	U213363	1.6
	DnaJ domain-containing protein	U229061	1.6
	Wound-induced protein Sn-1, vacuolar membrane	U215327	1.5
	Disease resistance response protein-related/ dirigent protein-related	U228401	-1.6
	Putative growth-regulating factor	U230293	-1.7
	Peptide methionine sulfoxide reductase	U213992	-1.7
	Glutathione transferase	U213824	-1.7
	Putative glutathione S-transferase T2	U212756	-1.8
	Gamma-thionin (plant defensins)	U214589	-1.9
	Hsp20.0 protein	U212695	-1.9
	Leucine-rich repeat	U220211	-2.3
	Cytosolic class II small heat shock protein HCT2	U216468	-2.5
	Class II small heat shock protein Le-HSP17.6	U216469	-2.6
	Mitochondrial small heat shock protein	U212696	-2.7
	Hsr201 protein, hypersensitivity-related	U216412	-2.9
	DnaJ homolog	U212657	-2.9
	Putative disease resistance protein	U227983	-2.9

[a] Unigene identifier from Sol genome network (SGN)

[b] Statistical analysis was done by using the F-test of the R/maanova package (Wu *et al.*, 2002). Only genes showing an adjusted p-value under 0.01 and a fold change higher than 1.5 and lower than -1.5 are presented.

Several other defense-inducible genes were modulated following sucrose retrieval from the medium (Table 3.5). Sugar deprivation slightly up-regulated the expression of a proteinase inhibitor I precursor (*PIN1*), a proteinase inhibitor II (*PIN2*), a wound-induced

protein (*SN-1*), a β-1,3-glucanase and a pathogenesis-related leaf protein 6 precursor (*P6*). Moreover, four genes associated to cold stress, namely two cold regulated proteins, the shock protein Soybean Gene Regulated by Cold-2 (*SRC2*), and the cold acclimation protein WCOR413-like, were induced following sugar retrieval. Two pathogenesis-related proteins (PR-5 protein), the Avr9/Cf-9 rapidly elicited protein and two elicitor inducible proteins were also induced by 1.7- and 3.7-fold when sucrose was retrieved from the growth medium. Inversely, two disease resistance proteins, the HSR201 protein, and a plant defensin (gamma thionins) were down-regulated 1.6-, 2.9-, 2.9- and 1.9-fold in sugar deprived plantlet. We also identified two glutathione S-transferases (*GST*) that were down-regulated. Both enzymes are Tau class GST well known to be highly responsive to stress conditions as they participate in the oxidative stress responses (Kampranis *et al.*, 2000; Kilili *et al.*, 2004).

3.5.8 Real-time PCR validation of microarray data

To support our microarray data, five genes showing differential expression were subjected to real-time PCR analysis (Fig. 3.1). These genes were selected according to their implication in various functional categories. Therefore, the mRNA transcript levels of four genes identified to be up-regulated genes under sugar deprivation were measured: trehalose-6-phosphate phosphatase (*TPP*), 1-aminocyclopropane-1-carboxylate synthase (*ACC*), ripening regulated protein DDTFR10/A (*DDTFR10/A*) and xyloglucan endotransglycosylase 2 (*SlXTH2*). Additionnally, one transcript shown to be down-regulated following sucrose retrieval was quantified: mitochondrial small heat shock protein (*MT-SHSP*). For all transcripts, the trends and fold change obtain through real-time quantitative PCR corroborated microarray data.

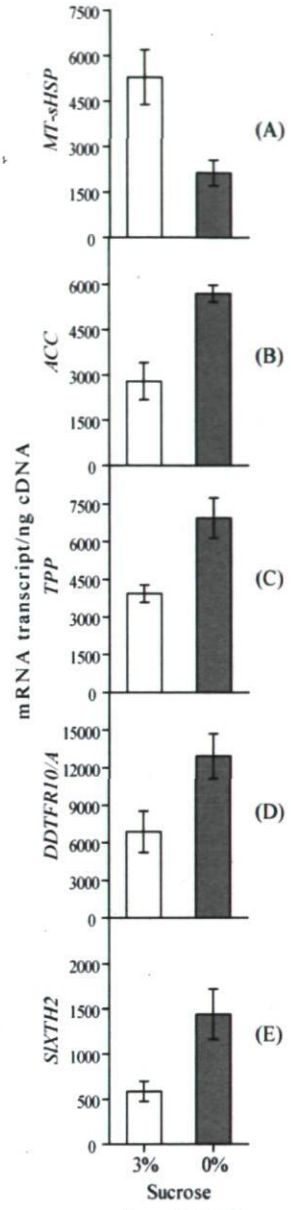

Figure 3.1 Validation of microarray expression data by real-time quantitative PCR (RT-PCR) analysis. Transcripts level of down-regulated (A) mitochondrial small heat shock protein (*MT-sHSP*, SGN-U212696), and up-regulated (B) aminocyclopropane-1-carboxylate synthase (*ACC*, SGN-U213523), (C) trehalose-6-phosphate phosphatase (*TPP*, SGN-U216105), (D) ripening regulated protein DDTFR10/A (*DDTFR10/A*, SGN-U214425) and, (E) xyloglucan endotransglycosylase 2 (*SlXTH2*, SGN-U220883) were obtained by RT-PCR and their pattern compared with microarrays data. The trend in mRNA transcript level was the same for RT-PCR and microarray analysis. Total RNA was extracted from plantlets cultured *in vitro* for a period of 20-d and transferred in liquid medium with (3%) and without (0%) sucrose for 24-h. Data represent mean expression of five measurement ± SE. Expression data were normalized on the basis of cDNA concentration. Results for all five mRNA transcripts were statistically significant at $p \leq 0.05$.

3.6 Discussion

3.6.1 Genes associated to cell wall and membrane are highly responsive to sucrose deprivation

Following transfer to acclimatization, *in vitro* plantlets are confronted to harsh conditions including higher light level, lower relative humidity, new atmosphere and sugar deprivation. To better understand the role of sugar upon acclimatization, a short-term study of the impact of sucrose retrieval in tomato upon transfer *ex vitro* was performed. For this, plantlets were maintained *in vitro* for 24 h under sugar-feed or -deprived conditions and submitted to gene expression analysis. A considerable percentage of responsive genes were involved in cell wall synthesis and turnover, and ethylene biosynthesis and responses. Somewhat unexpectedly, after 24 h of sugar deprivation, microarray data showed that the transcriptome of *in vitro* tomato plantlets readily adapts to the new condition but not through a sugar, lipid and amino acid catabolism, nor through an increase in photosynthesis or cell growth arrest responses.

The cell wall is a complex interlacement of cellulose, pectin, hemicelluloses and structural proteins. Variation in the expression of different enzymes involved in the biosynthesis of these complex polysaccharides can have major impacts on the cell wall properties. In plants, the biosynthesis of the primary cell wall requires the nucleotide sugar UDP-glucuronic acid (UDP-GlcA) for the production of the polysaccharide matrix, mainly composed of hemicellulose and pectin. The production of UDP-GlcA can be achieved by two different pathways (Kanter *et al.*, 2005). On the one hand, UDP-GlcA is produced by the enzyme UDP-glucose dehydrogenase (UGD). On the other hand, UDP-GlcA is also synthesized through the myo-inositol pathway. The conversion of glucose-6-phosphate to glucoronic acid (Glc-A) is therefore realized by the enzyme myo-inositol-1-phosphate synthase and myo-inositol oxygenase (*MIOX*). In our experiment, these two genes had a higher expression when plantlets were transferred to 0% sucrose. Interestingly, as an indicator of the relative expression level of the genes, database search for EST coding for *MIOX* and *UGD* showed that the former exceed the number of the latter by up to 10-folds, indicating a higher levels of expression of *MIOX* over *UGD* and possibly the prevalence of myo-inositol pathway for cell-wall synthesis in tomato (Kanter *et al.*, 2005). In addition,

the enzyme UDP-glucose-4-epimerase, responsible for the conversion of UDP-GlcA to UDP-galactose (UDP-GalA) was up-regulated upon sugar retrieval from the medium. UDP-galactose is a key sugar molecule involved in cell wall polysaccharide synthesis. These results suggest a stimulation of cell wall nucleotide sugar synthesis after 24h of sugar deprivation.

Under specific events such as elongation, secondary wall formation, senescence and cell wall turnover, the polysaccharide backbone of the cell wall is subjected to cleavage. These developmental processes are highly dependent on the activation of cell wall degrading enzymes. A considerable number of transcripts regulated under sugar deprivation are associated to cell wall degradation and turnover (Table 3.2). Specifically, the expression of a pectinesterase was enhanced upon sucrose retrieval. Highly esterified pectins are produced by the Golgi apparatus and delivered to the cell wall. At this level, the action of pectinesterase can increase cell wall rigidity by formation of pectate gel or increase cell wall hydrolysis as pectin degrading enzymes have more affinity for pectate. At the same time, a ß-galactosidase, involved in breakdown of ß-galactan links was up-regulated. This enzyme is known to participate in pectin degradation and formation (Gou *et al.*, 2007). Concomitantly, the transcripts from a ß-1,4-xylosidase, which are involved in the breakdown of hemicelluloses with a ß-1,4-xylose backbone, followed the same trend. In contrast, another xylosidase and a endo-β-1,4-D-glucanase gene, involved in xyloglucan and cellulose hydrolysis, were slightly down-regulated in sugar-deprived plantlets. These data are interesting as they point out the important regulation of cell wall genes by sucrose and suggest that pectin and hemicelluloses are submitted to increased cleavage under sugar deprivation. Furthermore, sugar depletion stimulated the expression of genes playing a critical role in cell wall reorganization and structure (Table 3.2). In this study, two of the most highly positively regulated genes were the xyloglucan endotransglucosylase-hydrolase 2 (SlXTH2, formerly LeXET2) and 5 (SlXTH5). These enzymes are potential cell walls loosening agents catalyzing the cleavage of a xyloglucan molecules and their subsequent linkage to other xyloglucans. XHT2 transcripts were reported to increase after rapid elongation and postulated to function in cell wall modification and/or strengthening (Catala *et al.*, 2001). Function of XHT5 is still nebulous, but a study has revealed that it is unlikely that XHT5 acts as primary cell loosening agents (Saladie *et al.*, 2006).

The up-regulation of two proline-rich proteins and an extensin-like protein (*NTEIG-C29*) suggest that structural proteins of the cell wall are also affected by sugar deprivation (Table 3.2). Extensins are structural proteins expressed following cell expansion as they are involved in increasing the tensile strength of the cell wall (Cassab, 1998). In particular, NtEIG-C29 was first identified in *Nicotiana tabacum* plants following treatment with oomycetes elicitor and was shown to be induced following wounding, SA and jasmonate treatments (Takemoto *et al.*, 2003). Therefore, the enhanced expression of these genes is coherent with a major rearrangement of the cell wall polysaccharides taking place under sugar deprivation.

Compelling evidences now show that sugar depletion have a direct impact on cell wall metabolism. Recent studies have demonstrated that there is a general increase in cell wall hydrolysing enzyme gene expression and decrease in pectin and hemicellulose in cell wall under sugar starvation in order to utilize energy reserves stored in the polysaccharide matrix (Lee *et al.*, 2004; Lee *et al.*, 2007a). Hence, we suggest that there is not only an increase expression of cell wall polysaccharide matrix hydrolysing enzymes, but that this process could initiate a global rearrangement in cell wall structure that might lead to cell elongation.

Deposition of lignin, a biopolymer fabricated mainly from coniferyl, coumaryl and sinapyl alcohol, three monolignols derived from the phenylpropanoid biosynthetic pathway, is characteristic of secondary wall formation and is responsible for increasing cell wall rigidity (Boerjan *et al.*, 2003). Lignin is also particularly important in response to pathogen infection. In our experiment, genes involved in lignin biosynthesis were regulated by sucrose. For example, the tomato *ELI3* gene code for a CAD enzyme, which catalyzes the final step of monolignols biosynthesis, was down-regulated in sugar-deprived plantlets. The *CS2* gene followed the same trend in expression. Chorismate, the end-product of chorismate synthase, is mostly used as a substrate for the synthesis of lignin in vascular tissues. Alternatively, chorismate is usually used to meet increased demand for suberin, lignin and salicylic acid upon pathogen infection (Macheroux *et al.*, 1999), but *CS2* was shown to be unresponsive to several stress elicitors (Gorlach *et al.*, 1995). Hence, these data suggest that lignin biosynthesis could be compromised following transfer to sugarless

conditions as depicted by the lower abundance of a lignin forming anionic peroxidase, ELI3 and CS2 transcript. Lignin biosynthesis is dependent on carbon availability and transfer to sucrose-free medium could lower its production (Rogers *et al.*, 2005). Still, it is not clear if lignin synthesis is repressed because a class III peroxidase was up-regulated during transfer to autotrophic conditions. This peroxidase was shown to be induced upon wounding and infection by *Phytophtora infestans* (Collinge & Boller, 2001). Thus, these results suggest that secondary cell walls biosynthesis tends to be repressed concomitantly to the increase in cell wall metabolism possibly to allow for more cellular elongation. Inhibition of lignin synthesis and wax deposition under sugar deprivation could seriously affect plantlets' capacity to confront adverse conditions imposed upon transfer *ex vitro*.

Cell membrane-related genes were also susceptible to sucrose level. The cell membrane is a highly plastic barrier that responds to environmental cues and internal signal by modifying its composition. Plantlets transfer from 3% to 0% sucrose caused an up-regulation of the expression of three desaturases, and inversely one was down-regulated. These enzymes are often involved in drought, salt and heat tolerance but also physiological events such as cell elongation and cell mitosis (Gou *et al.*, 2007). Notably, the FAD2 gene was among the up-regulated gene in plantlets under sugar deprivation. FAD2 has recently been shown to be developmentally regulated as its mRNA transcript increased during leaves expansion in *Nicotiana tabacum*. Functionally, FAD2 is postulated to be involved in the production of linoleic (18:2) and linolenic acid (18:3) necessary to the development of thylakoïd membranes (Yang & Xu, 2007). We also reported an increase expression of the tomato DES-1 gene coding for a sphingolipid desaturatase. Sphingolipids play a critical role in cell signaling as their desaturation at the Δ4 position by the enzyme DES was shown to be essential for the cell cycle transition between G2/M during spermatogenesis in *Drosophila melanogaster* and cell mitosis, differentiation and apoptosis in mammalian cells (Napier *et al.*, 2002). A similar DES-1 gene from tomato was identified through homology with other DES family members, but no functional characterization was provided (Ternes *et al.*, 2002), but probably regulates cell division. In plants, sphingolipids signaling are believed to play a role in stomatal regulation, particularly during drought stress, in *Commelina communis* (Ng *et al.*, 2001) and in programmed cell death in *Arabidopsis* (Shi *et al.*, 2007). Lipoxygenases (LOX) are well known for their involvement in plant growth

and development and for the biosynthesis of the lipid-derived phytohormone jasmonic acid (JA) (Feussner & Wasternack, 2002). Sugar deprivation response included the down-regulation of a *LOX* gene. Here, the LOX gene may function in cell elongation or in defense response (Siedow, 1991). These results indicate that sucrose deprivation induce a coordinated modulation of lipid biosynthesis leading to important modification in cell membranes.

3.6.2 De-repression of ethylene biosynthesis and transcriptional regulation by sucrose deprivation

Interestingly, key genes closely related to ethylene biosynthesis and signaling were up-regulated after sugar deprivation. Ethylene and polyamine biosynthesis both uses S-adenosylmethionine (SAM) as a precursor. The down-regulation of SAM decarboxylase (*SAMDC)* and the up-regulation of *ACC* synthase expression suggests that SAM is mainly directed toward biosynthesis of ethylene to the disadvantage of polyamine biosynthesis. Drastic increase in ethylene production was previously reported in potato SAMDC antisense mutant (Kumar *et al.*, 1996). These results are also confirmed by the up-regulation of several transcriptions factors belonging to the NAC, AP2/EREBP, and bHLH family known to respond to ethylene. More precisely, the NAC domain containing protein 2 (*NAC2*), an AP2 domain transcription factor, the transcriptional activator CBF1 (*DREB1B*), and the ripening-regulated protein *DDTFR10* genes, homologue to several ethylene-responsive elements binding protein, were up-regulated in this experiment. An *Arabidopsis* mutant study revealed that *AtNAC2* expression was tightly linked to ethylene and auxin signaling and overexpression resulted in enhanced lateral root development (He *et al.*, 2005). In the same way, the transcription factor CCR4-associated factor (*CAF1*) was up-regulated by sugar deprivation. *CAF1* gene was also shown to be regulated by ethylene in *Arabidopsis* (Van Zhong & Burns, 2003). Recent studies have identified the CAF1 as an important transcription regulator that affects plant growth and development by altering the expression of many genes involved in cell wall biogenesis (Sarowar *et al.*, 2007). In tomato, over-expression of the *Capsicum annuum* CCR4-associated factor (*CaCAF1*) was identified to increase the cell wall and cuticle thickening, mesophyll cell length and the expression of cell wall modifying enzyme such as pectinesterase, cellulose synthase, peroxidase and fatty acid desaturase. Furthermore, ethylene may act synergistically with

other hormones in the observed response. Notably, auxin induced *IAA17/AXR3* transcription factor was shown to alter transcript levels of many genes associated to cell wall biosynthesis and degradation (Overvoorde *et al.*, 2005) and therefore its up-regulation may be involved in a cascade of events leading to cell wall synthesis perturbation by sucrose. These results suggest that sucrose deprivation have a profound effect on ethylene signaling pathway and confirm the importance of sugar in the repression of ethylene biosynthesis and response.

More and more evidence suggest that sugars act as a repressor of ethylene action (Price *et al.*, 2004). For instance, analysis of different *Arabidopsis* mutants revealed a negative interaction between glucose and ethylene. Indeed, glucose insensitive (*gin)* mutant has a phenotype similar to ethylene-treated wild type plants or constitutive ethylene signalling mutant phenotype. Also, the ethylene insensitive mutant (*etr1*) was demonstrated to have a phenotype similar to glucose oversensitive mutant (*glo*) (Yanagisawa *et al.*, 2003; Zhou *et al.*, 1998). Additionally, glucose was also shown to acts on ethylene-insensitive3 (EIN3), a transcription factor involved in ethylene signal-transduction, by controlling its degradation. Moreover, it's noteworthy that a microarray analysis of ethylene responses in *Arabidopsis* reported the regulation of several stress-related, transcription factors, and cell wall protein transcripts (Van Zhong & Burns, 2003) as described in our experiment. Therefore, the up-regulation of several ethylene-regulated genes under sugar deprivation suggests that exogenous sucrose interfere with ethylene signaling. Hence, it is not surprising that plantlets transferred from 3% to 0% sucrose display many physiological changes including senescence, cell expansion, and induce defense responses. Recently, sucrose was shown to delay senescence in carnation petals by hampering the up-regulation of genes involved in ethylene signaling and biosynthesis, cell wall degradation and defense (Hoeberichts *et al.*, 2007) e.g. the hypersensitivity-related (HSR) 201 protein, a PR5 protein, and a Avr9/Cf-9 rapidly elicited protein. Similarly, our result showed that two pathogenesis-related proteins (PR-5 protein), and Avr9/Cf-9 rapidly elicited protein were up-regulated and the HSR201 protein was down-regulated under sugar deprivation. Ethylene also play a determinant role in plant cell elongation (Pierik *et al.*, 2007), in cotton fiber cells (Shi *et al.*, 2006), in *Brassica juncea* leaf (Khan *et al.*, 2008), in *Arabidopsis* hypocotyl (Smalle *et al.*, 1997), and in *Triticum aestivum* seedling internodes

(Suge *et al.*, 1997). Ethylene effect on cell expansion is not unequivocal and low ethylene can increase cell elongation while high ethylene can reduce leaf expansion (Fiorani *et al.*, 2002). Besides, ethylene is also an important regulator of the shade-avoidance response. This photomorphogenetic response allows plant to adjust their growth and development to adapt to low light conditions and is mainly characterized by the induction of stem and petiole growth (Pierik *et al.*, 2007). In micropropagation, plantlets are submitted to low light intensity (< 200 µmol photons m^{-2} s^{-1}) and their development relies almost solely on exogenous sucrose. That being so, we observed the down-regulation of *PHYB1* gene under sugar deprivation. Earlier studies demonstrated that *PHYB1* overexpression restricted elongation in tomato and mutant analysis showed that *PHYB1* is involved in the shade-avoidance response (Husaineid *et al.*, 2007; Weller *et al.*, 2000). Sugars were previously shown to interact and modulate phytochrome sensing and signaling pathways (Dijkwel *et al.*, 1997; Short, 1999; Thum *et al.*, 2003). This result suggests that sucrose retrieval regulate phytochrome and ethylene-related genes expression which could initiate a shade-avoidance response. Finally, ethylene is also closely involved in defense induction under various biotic and abiotic stresses (Wang *et al.*, 2002). Several genes regulated by sugar deprivation involved in plant defense were also shown to be regulated by ethylene. Among these, two extensins, a β-1,3-glucanase, a protease inhibitor (PIN2) (Van Zhong & Burns, 2003) and, a wound-induced protein (SN-1) (Hermsmeier *et al.*, 2001) were up-regulated and LTP's were variably modulated by sucrose retrieval. Moreover, the up-regulation by sugar deprivation of two elicitor-activated genes and two cold-regulated proteins may also be attributable to ethylene response. Important interactions exist between senescence, cell expansion, shade avoidance, and defense responses as all these processes are involved in the induction of genes related to cell wall breakdown and stress responses. Further studies will be needed to fully characterize the physiological implication of increase ethylene response observed in plantlets subjected to sugar deprivation and it implication upon plantlet acclimatization. It is possible to envisage that acclimatization problems experience by *in vitro* plantlets could be linked to the stimulation of senescence through ethylene response. Spike in ethylene biosynthesis and response following sucrose deprivation could interfere with the initiation of process essential to plantlet acclimatization such as the

catabolism of reserve and the development of autotrophy which would hamper plantlet survival.

3.6.3 Sugar deprivation inhibits genes involved wax deposition

Acclimatization success of *in vitro* plantlets is highly dependent on their capacity to restrict water lost. When transferred from *in vitro* water saturated atmosphere to *ex vitro* lower air humidity, plantlets are subjected to increased vapour pressure deficit (VPD) and thus to increased transpiration. In this context, wax deposition in plantlets is of particular interest as it may limit non-stomatal water lost. Data analysis showed that *CER1* was down-regulated under sugar deprivation. *CER1* is believed to be involved in synthesis of long chain lipids necessary for wax formation (Samuels *et al.*, 2008). The analysis of *CER1* mutant revealed a low wax deposition of only 13 % of the wild type. This result was also corroborated by the lower expression of a cuticular protein. Therefore, sugar deprivation may lead to decrease wax deposition and is even more detrimental to acclimatization success. More research is needed to unravel the role of sugar on cuticle formation.

Plant LTPs fulfill several biological roles: i.e. growth and development, defense, and cuticle deposition, cell wall extension and plant signaling (Nieuwland *et al.*, 2005). Here, we report the specific modulation of multiple LTPs by the removal of sugars from the culture medium. The localization and function of the two up-regulated and four down-regulated LTPs have not been characterized until now, but results obtained suggest that they could be involved in cell wall metabolism and/or defense responses. For example, the down-regulated *TSW12* gene has been shown to encode a LTP particularly responsive to salinity and heat shock in stems of tomato plants, but its function still remains hypothetical (Torres-Schumann *et al.*, 1992). Additionally, sugar deprivation lead to the down-regulation of two ABC transporter genes. Previously, an ABC transporter (CER5) and certain LTPs were believed to be involved in wax trafficking through the plasma membrane and from the cell wall to the cuticle, respectively (Samuels *et al.*, 2008). In the light of the data presented, the down-regulation of two ABC transporters and several LTP mRNA suggest that they could be involved in cuticle deposition. Past studies on *in vitro* plantlet leaf structure have reported reduce cuticle thickness (Pospisilova *et al.*, 1999; Sutter & Langhans, 1982). The impact of sugar level on cuticle formation is largely unknown and

these results suggest that sugar deprivation further accentuate the decrease in wax deposition in *in vitro* plantlets. Hence, a sudden decrease in wax production upon plantlet transfer *ex vitro* could compromise their capacity to survive under lower relative humidity. Considering that *ex vitro* formed leaf show normal cutin and wax deposition, the decrease in cuticle formation is probably only transitory (Hazarika, 2006) and only affects *in vitro* leaves.

3.6.4 Sucrose deprivation modulates genes involved in primary metabolism

Energy remobilization must occur rapidly when plants are confronted to sugar deprivation. For example, sugar starvation in suspension cell culture was shown to induce a rapid stimulation of genes linked to sugar remobilization and transport, and to inhibit carbohydrate storage and utilization (Wang *et al.*, 2007). We also report the increase transcript level of three sugar transporters which indicate that plantlets initiate the re-distribution of carbon throughout the plant upon sugar retrieval. These results are also coherent with those presented by Price *et al.* (2004), who observed that glucose treatment inhibited the expression of multiple sugar transporters. The activation of sugar transporters could also account for the reallocation of sugar toward cell wall expansion or modification (Lee *et al.*, 2007b).

In contrast, we observed the down-regulation of a *UQ*, a *CYT BC1*, a *CYT C*, and a frataxin protein-related gene involved in the ETC from mitochondria. These results suggest that a decrease in respiratory processes occurs within 24h after transfer to 0% sucrose, and therefore that plantlets are just beginning to suffer from sugar depletion. Surprisingly, the photosynthetic apparatus of *in vitro* plantlets did not respond to sugar removal since only two genes were up-regulated. However, sugar deprivation increased the expression of a *TPS* and a *TPP* gene, which are known to function in trehalose biosynthesis. Trehalose plays a pivotal role in regulating glycolysis and higher expression level of *TPS* and *TPP* has been postulated to favor carbohydrate utilization over storage under sugar starvation (Contento *et al.*, 2004). This observation is coherent with the down-regulation of a starch synthase, involved in sugar storage, upon sucrose retrieval. Recent results identify trehalose as an important regulator of many cellular processes including sugar metabolism, cell wall

and membrane, stress-response, and plant growth and development (Bae *et al.*, 2005b; Eastmond *et al.*, 2003). Moreover, exogenous trehalose up-regulated several transcripts related to ethylene signaling pathway (Bae *et al.*, 2005b).

The GABA shunt was particularly responsive to sugar deprivation. Growing body of evidence demonstrates the participation of the GABA shunt in carbon and nitrogen partitioning and its potential role as a key modulator of plant metabolism following sucrose retrieval (Fait *et al.*, 2008) On the one hand, higher GAD expression, which convert cytosolic glutamate to GABA, may participate in the storage of excess nitrogen stemming from protein degradation in sugar-deprived plantlets as reported in tomato fruit (Baldet *et al.*, 2002). On the other hand, lower GHBDH expression could favor replenishment of the TCA cycle with succinic semialdehyde (SSA) produced by the up-regulated GAD gene under sugar deprivation. In this context, GABA could maintain sustained respiration and thus, could be involved in metabolic by-pass in sugar-deprived plantlets. These results confirm that *in vitro* plantlets must initiate important compensatory processes after 24h of sugar deprivation to adjust carbon availability and consumption. Protein turnover is crucial under fluctuating environmental conditions. In sugar-deprived tomato plantlets, we identified a preferential up-regulation of genes involved in protein degradation, such as E3 ubiquitin ligase family and the *APG8H* gene, involved in autophagy. These results confirm those of Osuna *et al* (2007) who reported the down-regulation of several E3 ligase following sucrose addition and with Contento *et al.* (2004) who reported increased expression of *APG8* gene and autophagy in suspension cell under sucrose starvation, suggesting an initiation of nutrient recycling (Thompson *et al.*, 2005). Genes associated to protein synthesis, such as ribosomal proteins, were variability regulated under our conditions. Unexpectedly, genes associated to amino acid biosynthesis and degradation were globally induced and repressed, respectively. This is somehow contradictory to data obtained by Price et al. (2004), who reported the up-regulation of genes involved in amino acid catabolism and down-regulation of amino acid biosynthesis. Such discrepancy can be explained by differing experimental set-up, as we have studied 20 days-old tomato plantlets in opposition to Price's group who opted for whole young *Arabidopsis* seedling. These observations suggest a tight regulation of protein synthesis and proteolysis in carbon-deprived plantlets after 24h. The increased protein catabolism and amino acid anabolism

could also be explained by increased degradation of unneeded proteins and synthesis of proteins involved in adaptation to sugar deprivation. It is interesting to note that the *ERF1* gene, involved in translation termination, was induced following sucrose retrieval. Mutant analysis in *Arabidopsis* revealed that cosuppression of *ERF1* resulted in reduced internode elongation (Petsch *et al.*, 2005). Hence, this result supports the involvement of exogenous sucrose in the regulation of cell expansion. Thus, these results suggest *in vitro* sugar-deprived tomato plantlets show no strong needs toward massive protein degradation, and increased amino acid catabolism and repression of amino acid biosynthesis. Breakdown of lipid to sucrose also occurs under starvation (Contento *et al.*, 2004). To be converted into ATP, lipids are successively subjected to lipolysis, β-oxidation, glyoxylate cycle, and gluconeogenesis pathway. The up-regulation of two lipases genes and the coordinated up-regulation of gluconeogenesis/ glyoxylate cycle, suggest that lipid catabolism is increased under sugar deprivation.

3.6.5 Hormone and Stress Response

Sucrose retrieval specifically modulated several genes involved in hormone metabolism. The induction of multiple auxin-regulated genes, and an Aux-IAA and auxin-induced TCP transcriptions factors suggest that the auxin signaling pathway is induced upon transfer to sucrose free medium. Modulation of auxin-responsive genes after sucrose was retrieved from the medium could be a consequence of the stimulation of ethylene signaling pathway. As reported before, auxin-regulated genes and possibly auxin biosynthesis were activated after treatment with exogenous ethylene (Van Zhong & Burns, 2003). Gene expression analysis revealed the down-regulation of two genes associated to gibberilic acid synthesis. The geranyl diphosphate synthase gene codes for an enzyme involved in the early steps of the terpenoid pathway responsible for the production of geranyl diphosphate (*GPP*), which has been recently associated to the gibberellins biosynthetic pathway (van Schie *et al.*, 2007). GA20OX1 is an important regulatory enzyme in the final steps of the gibberellic acid biosynthetic pathway. Gibberellins and/or its precursors are synthesized primarily in the apical bud and young leaves and distributed throughout the plant via the phloem. These results suggest that reduce development under sugar deprivation could lead to lower gibberellins production and transport. Although,

phytohormones are often used in tissue culture to induce growth, the *in vitro* media used here did not contain any growth regulator.

Although several stress-related genes were up-regulated by sugar deprivation, HSPs family gene followed an opposite trend. In particular, lower expression of *TSW12* and of multiple sHSPs upon transfer to autotrophic conditions indicate that sucrose deprivation down-regulated specific defense-related genes involved in heat stress. The exact meaning of such responses is still obscure. Sucrose could enhance plant metabolism and therefore increase needs for protein folding by HSP (Price *et al.*, 2004). Inversely, *DNAJ* genes were up-regulated by sugar deprivation. DNAJ proteins act as a co-chaperone of DNAKs (Hsp70), which were shown to fulfill important functions in protein refolding, import, translocation, and proteolytic degradation under normal and stressful environmental conditions (Wang *et al.*, 2004). The increase gene expression of *DNAJ* proteins was previously reported in *Arabidopsis* suspension culture upon sugar starvation and postulated to favor proper chloroplast biogenesis under nutrient stress (Contento *et al.*, 2004).

3.7 Conclusions

In the light of the results presented here, we hypothesize that exogenous sucrose *in vitro* inhibits the biosynthesis of ethylene and its retrieval from the growing media initiates a signaling cascade possibly leading to modification in cell wall properties. Furthermore, changes in transcription factors, trehalose metabolism, GABA shunt and protein turnover suggest that important pathway involved in metabolism adjustment to changing sugar supply are initiated. The alteration of plantlets anatomy and physiology associated to sugar deprivation can have major impact on plantlets survival *ex vitro*. Lower expression of enzymes involved in epicuticular wax synthesis, lignin, and perturbation of cell wall properties could certainly be detrimental when plants undergo acclimatization in low humidity conditions and higher light intensity. Surprisingly, plantlets did not adapt photosynthetically after 24 h. Thus, this result may imply that these changes are taking place on a longer time-scale and may thus explain to a certain extent the difficulty *in vitro* plantlets have to adapt to the rapid transfer to acclimatization. Time-course experiments

over a longer period would increase our capacity to characterize the metabolic adaptation of *in vitro* cultured plantlets through the acclimatization step.

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Chapitre 4 : Proteomic approach toward a better understanding of the impact of exogenous sucrose in *in vitro* tomato (*Solanum Lycopersicum*) plantlets

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4.1 Résumé

Le statut en carbone des plantes est un signal crucial pour la coordination de la croissance et du développement et l'ajout de saccharose exogène dans le milieu de culture tissulaire entraîne une modification importante de la physiologie, de l'expression génique et de l'accumulation protéique chez les plantules. Précédemment, différentes études sur l'effet du saccharose sur le profil d'expression génique ont été réalisées, cependant, peu de données sont disponibles sur l'impact des sucres sur le protéome des plantes. Des plantules de tomate (*Solanum lycopersicum*) ont donc été cultivées *in vitro* pendant vingt jours en présence (3%) ou absence (0%) de saccharose afin de mesurer l'effet à long terme du saccharose exogène sur le métabolisme des plantules. L'impact du saccharose exogène sur l'abondance des protéines des feuilles de plantules de tomate a été mesurée par électrophorèse bidimensionnelle (2-DE). Dix-sept protéines présentant une accumulation différentielle ont été identifiées par spectrométrie de masse. Nous avons observé une augmentation de l'abondance de protéines impliquées dans l'élongation, le repliement et la dégradation des protéines, la synthèse de nucléotide et le transport mitochondrial chez les plantes mises en présence de saccharose. À l'inverse, des protéines impliquées dans la chaîne de transport d'électron photosynthétique et la fixation du carbone, et une peroxydase ont affiché une diminution de leur abondance en présence de saccharose exogène. Les paramètres photosynthétiques mesurés concordent avec la modification de l'accumulation des protéines observées. La Cu/Zn superoxide dismutase et la glutathione peroxydase ont présenté une diminution et une augmentation de leur abondance, respectivement. Ces résultats montrent une implication majeure du saccharose exogène dans la régulation de la prolifération cellulaire, la photosynthèse et le mécanisme de défense contre les dérivés réactifs de l'oxygène. Tout compte fait, les changements métaboliques induient par le saccharose exogène devraient être atténués avant de procéder au transfert des plantules *ex vitro*.

4.2 Abstract

The plant carbon status is a crucial cue for the coordination of plant growth and development and the addition of exogenous sucrose in tissue culture causes important modification in plantlet physiology, gene expression and protein accumulation. Although numerous studies are available on the effect of sucrose on gene expression profile, limited data is available on the outcome of sugar on the plant proteome. Tomato (*Solanum Lycopersicum*) plantlets were cultured *in vitro* for twenty days with (3%) or without (0%) sucrose to measure the long-term effect of exogenous sucrose on plantlet metabolism. The impact of exogenous sucrose on leaf of tomato plantlets protein abundance was measured using two-dimensional electrophoresis (2-DE). Seventeen spots showing differential accumulation were identified by mass spectrometry. In sucrose-fed plantlets, proteins which function in protein elongation, folding and degradation, nucleotide synthesis and mitochondrial transport increased in abundance. Inversely, protein involved in photosynthetic electron transport chain and carbon dioxide fixation, and a peroxidase decreased in abundance in presence of exogenous sucrose. Plantlet photosynthetic measurements concurred with the modification in protein abundance. The chloroplast Cu/Zn superoxide dismutase and glutathione peroxidase proteins decreased and increased in abundance, respectively. These results support an important implication of exogenous sugar in cell proliferation, photosynthesis and reactive oxygen species scavenging mechanisms *in vitro*. The modification in plant metabolism induced by exogenous sucrose should be mitigated before transferring plantlet *ex vitro*.

Key words: plant, *in vitro*, tissue culture, micropropagation, sugar, proteomic, photosynthesis, protein synthesis, *Solanum Lycopersicum*, acclimatization

4.3 Introduction

Plants, as sessile organism, are force to adapt to the environment where they grow. As may happen in natural or artificial growing condition, as in the case in micropropagation, plants are confronted to wide diversity of environmental conditions. In order to adjust to the constantly changing environments, plants have developed complex regulatory systems. The plasticity of the plantlets has its limits and plantlets may suffer irreversible damages leading to death if growing under highly unfavourable and rapidly shifting environment. For example, when *in vitro* grown plantlets are transferred to the greenhouse and field under uncontrolled conditions, they can rapidly die. Micropropagation is an *in vitro* technique used for propagating several agriculture crops and ornamental species, regenerate recalcitrant, endangered species and genetically-modified plants. Although, *in vitro* culture allows the mass production of millions of disease-free plants each year's, efficient acclimatization remains a serious problem for many micropropagated species.

In vitro culture conditions are characterized by unique features such as poor gas exchange, low CO_2, high relative humidity, and low light intensity which are assumed to have negative impact on photosynthesis. To partially compensate for these limiting growth conditions, sugar is added to the micropropagation medium. Plantlet cultured in presence of exogenous sucrose, i.e. in photomixotrophic conditions, often exhibit enhanced growth rate, dry weight, rooting, and improved establishment (Hazarika, 2003). However, the use of sugar *in vitro* is not without any inconveniences and often leads to contrasting results. In fact, exogenous sugars have been shown to have negative effects on plantlet photosynthetic capacity of several species such as *Solanum tuberosum* (Wolf *et al.*, 1998), *Solanum lycopersicum* (Kubota *et al.*, 2001), *Coffea arabusta* (Nguyen *et al.*, 1999), and *Cocos nucifera* (Fuentes *et al.*, 2005). For instance, the lower photosynthetic capacity of photomixototrophically grown plantlet was previously attributed to decreased Rubisco activity (Desjardins *et al.*, 1993). Inversely, other studies demonstrated the positive effect of sugar on photosynthesis of *Vitis vinifera* (Fila *et al.*, 1998) and *Nicotiana tabacum* (Ticha *et al.*, 1998). Conflicting data are also observed concerning the role of sugar with respect to plantlets' capacity to acclimatize *ex vitro* and have led to two general hypotheses.

Firstly, exogenous sugar serves to increase reserves and improves tolerance of plantlets to the severe conditions *ex vitro*. On the contrary, sugar affects photosynthesis negatively and plantlets cultured with exogenous sugar are unable to develop autotrophy quickly enough upon transfer *ex vitro* to survive. Recently, a microarray expression analysis of *in vitro* and *ex vitro* plantlets revealed an important perturbation of sink strength, and the up-regulation of specific defense responses in *in vitro* plantlets (Dubuc, 2010). This study also revealed interesting results on the impact of *in vitro* culture conditions on plantlets abnormal physiology and on the reactive oxygen species (ROS) pattern in the different cell compartments. Therefore, rapid transition from photomixotrophy to photoautotrophy upon acclimatization could lead to important oxidative stress and ultimately compromise the viability of plantlets.

Several recent studies have proved the far-reaching effect of sugar on developmental processes. In the last ten years, a great deal of knowledge on the impact of sugar on plant metabolism has been obtained through expression analysis. In particular, sugar abundance lead to the up-regulation of gene involved in glycolysis, respiration, signal transduction, transport, hormone signaling, DNA, RNA and protein synthesis, and stress response and down-regulation of gene involved in photosynthesis, chloroplast protein metabolism and sugar, amino acid and lipid catabolism (Osuna *et al.*, 2007; Price *et al.*, 2004). Plant carbon status is a crucial cue to the coordination of growth (Smith & Stitt, 2007). In *Arabidopsis* suspensions culture, sucrose regulation of D-type cyclin, CycD2 and CycD3, showed the importance of sugar in the control of cell division (Riou-Khamlichi *et al.*, 2000). More recently, the regulation of the expression of ribosomal proteins, which are strongly link to cell proliferation and sugar abundance, was attributed to the sugar-induced nucleolin-1 gene in *Arabidopsis* (Kojima *et al.*, 2007). Therefore, a close relationship exists between the cell nutritional state, protein synthesis, and cell proliferation.

Hence, the paucity of data on *in vitro* plantlet physiology and biochemistry is an important constraint and further research with high-throughput approach is needed to shed new light on the impact of carbon availability *in vitro* and at acclimatization. Although, transcriptomics studies have highlighted fundamental aspect of plant responses to sugar, only the short term effect ($\leq$ 48h) of sugar resupply or deprivation was measured.

Moreover, up to now, the impact of exogenous sucrose on large scale plant protein abundance has not been measured. Therefore, we carried a proteome study of tomato plantlet grown under two different sugar concentrations *in vitro* for 20 days using two-dimensional electrophoresis (2-DE) combined to mass spectrometry. Result shows that sucrose deeply interfere with plantlet photosynthesis, cell ROS scavenging protein, and cell growth and development. We discussed about possible implication of these data on plantlets acclimatization. As a model species in the study of carbohydrate metabolism, and due to the large protein database of this Solanaceae species, tomato was chosen for this project.

4.4 Materials and methods

4.4.1 Plant material and growth conditions

Tissue cultured tomato (*Solanum lycopersicum* L.) plantlets were propagated by internode cuttings at monthly intervals on MS medium supplemented with 8% (w/v) bacto-agar (BD Diagnostic System, Sparks, MD, USA), and 3% (w/v) sucrose. For the experiment, axillary apices were transplanted solely in stacked double magenta vessels (Sigma, Oakville, ON, Canada) and grown for 20-d on solid MS media containing either 3% or 0% (w/v) sucrose, respectively. Two sides from the upper magenta vessel were perforated to add Milliseal (Millipore, Billerica, MA, USA) gas-permeable filter disks. Plantlets were grown under 180 µmol photons m^{-2} s^{-1} PAR, 16-h photoperiod, and 23/20°C day/night temperature in a growth chamber (Conviron, Winnipeg, MB, Canada). After 20-d, plantlets second leaf was harvest, immediately frozen in liquid nitrogen, and stored at -80°C.

4.4.2 Protein extraction from leaf tissue

Tomato second leaf tissue were ground to fine powder using a mortar and pestle in presence of liquid nitrogen. Proteins were precipitated by resuspending the powder in 1 mL of a solution containing 10% trichloroacetic acid (TCA), 0.07% (v/v) 2 -mercaptoethanol in acetone for 2-h at -20°C. Precipitation was completed by centrifugation for 25 min at 4°C. Pellets were washed seven times in 10% double distilled water, and 0.07% (v/v) 2 -mercaptoethanol in acetone. Washes were executed for 10 min at -20°C and followed by a

25 min centrifugation at 4°C. Pellets were solubilised in 600 µl of a buffer containing 8M urea (w/v), CHAPS 2% (v/v), IPG buffer pH 3-10 and 60 mM dithiothreitol (DTT). Protein were further solubilised for 1-h in a sonication bath (Bransonic model 1510, Branson, Danbury, CT, USA) followed by a resting phase of 1-h at 32°C in a dry bath. Recovered proteins were quantified using Bradford protein assay (Bradford, 1976) as described in the manufacturer's protocol (Bio-Rad, Mississauga, ON, Canada). Proteins (200 µg) were dissolved in solubilisation buffer to obtain a final volume of 270 µL.

4.4.3 Two-dimensional electrophoresis

Total isolated proteins (200 µg) were loaded on a 13 cm immobiline DryStrip gel with a linear pH 3-10 gradient (GE Healthcare, Buckinghamshire, England). Active rehydration of DryStrip gel was carried out for 12-h at 30 V using Ettan IPGphor isoelectric focusing unit (GE Healthcare). Isoelectric focusing (IEF) was realized using voltage-gradient steps i.e. 100 V (1-h), 500 V (1-h), 1000 V (1-h), 5000 V (1-h), and 8000 V (to reach 19000 V-h). Immobiline DryStrip gel equilibration was performed using DTT, and iodoacetamine, for first and second equilibration, respectively, according to the manufacturer's handbook (GE Healthcare). Second dimension was realized running IPG strip on 12% polyacrylamide gel in Protean II XL system (Bio-Rad). Gels were stained with GelCode blue reagent (Pierce, Rockford, IL, USA). Four replicates consisting of four different tomato plantlets were realized for both treatments.

4.4.4 Image acquisition, analysis, and protein identification

2-DE gels were digitalized at 300 dpi with ImageMaster LabScan v.2003.01. Spot detection, gel to gel matching, and statistical analysis were performed using Phoretix Expression software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Background was substracted using the method of non-spot. Prior to analysis, spot volume data were normalized using the method of "total of spot volume". For this method, the volume of each spot is divided by the total volume of all the spots. Each spot was subjected to a statistical analysis with ANOVA ($p<0.05$). Sixteen proteins with differential accumulation in both treatments were cut from the gel manually. Spots were submitted to trypsin in-gel digestion on a MassPrep robotic workstation (PerkinElmer, Waltham, MA, USA) and peptides separated by a LC-MS/MS QTOF micro (Waters, Milford, USA) at Genome

Quebec Innovation Centre (McGill University, Montreal, Canada). Protein identification was realized with the MASCOT software (Matrix Science) by interrogating the NCBInr database (taxonomy viridiplantae) and EST others database (taxonomy "other green plants"). Other parameters for MASCOT search were: carbamidomethylation of cysteine residues (fixed modification), oxidation of methionine (variable modification), one missed cleavage and 0.3 Da mass tolerance. The spot 322 was identified by SELDI-TOF MS as described by Badri *et al.* (2009).

4.4.5 Determination of photosynthetic parameters

Light response curves were established using a portable infrared gas analyzer, LI-6400 (Li-Cor, Lincoln, NB). Measurements were taken at least 2 h after the beginning of the photoperiod. Leaf net photosynthetic rate was measured at a CO_2 concentration of 400 µmol m^{-2} s^{-1} for seven different levels of light (1000, 500, 200, 100, 50, 20, 0 µmol photons m^{-2} s^{-1}) for three biological replicates. The Photosyn Assistant software (Parsons & Ogston, 1999) was used to establish an A/Q curve and to estimate photosynthetic parameters, notably apparent quantum efficiency (Φ), light compensation point, apparent respiration (Resp) and light saturated maximum (Amax).

4.5 Results

4.5.1 Two-dimensional electrophoresis, identification and annotation of differentially accumulated protein

Tomato axillary apices were cultured *in vitro* on sucrose-free or sucrose-supplemented MS medium to investigate the impact of *in vitro* culture conditions on plantlets' protein accumulation. The comparison of protein profile of *in vitro* photomixotrophic and photoautrophic plantlets by two-dimensional electrophoresis (2-DE) allowed the quantification of approximately 680 GelCode stain proteins (Fig.4.1). Proteins on the 2-DE gel were dispersed across the 3-10 pI range and weighed between 8 and 180 kDa approximately. From the protein present on the gels, 65 proteins exhibited statistically significant ($p \leq 0.05$) difference in terms of abundance, of which 58 and 7 increased or decreased in content, respectively. Seventeen proteins with a sufficient spot volume were selected for Peptide Mass FingerPrint analysis. All spot subjected to LC MS/MS analysis were identified (Table 4.1). Of these proteins, five and eleven showed decreased or

increased accumulation, respectively, under photomixotrophic conditions (Table 4.1). Moreover, one protein, that was only present in plantlets grown under photoautotrophic condition, was identified (Table 4.1). Protein identification was performed by interrogating NCBInr and NCBI EST database from viridiplantae. The seventeen proteins were identified in Solanaceae species.

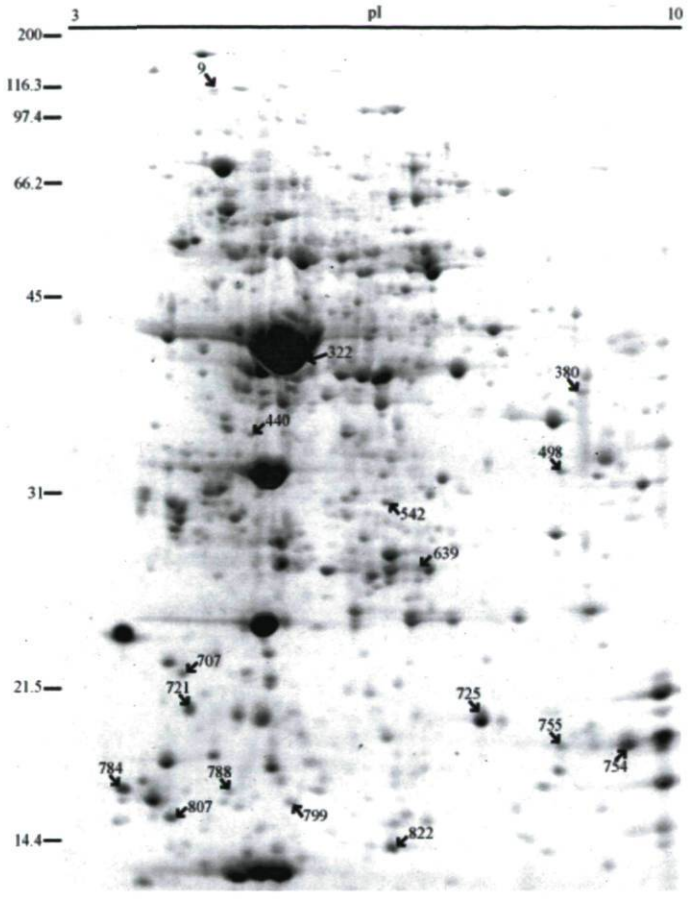

Figure 4.1 Two-dimensional gel separation of *Solanum Lycopersicum* leaf proteins cultured *in vitro* under photoautotrophic conditions. Total isolated proteins (200 µg) were loaded on an immobiline DryStrip gel with linear pH 3-10 gradient followed by 12% SDS-page. 2-DE was stained with GelCode blue reagent. Arrows and numbers indicate the protein with differential accumulation in response to sucrose level.

Table 4.1: Proteins with increased and decreased content in tomato leaf in response to exogenous sucrose *in vitro*.

Spot number	Protein identification	Organism	Accession number	M_r/pI		Score[b]	Sequence coverage[c]
				Theoretical	Observed[a]		
Protein that increased in presence of sucrose							
440	20S proteasome alpha 6 subunit	*Nicotiana benthamiana*	gil22947842	30.1/5.1	36/4.9	370	24%
498	Porin I	*Solanum tuberosum*	gil515358	29.4/7.8	33/8.2	552	25%
542	homolog to NAD-dependent epimerase/dehydratase	*Solanum lycopersicum*	gil60108927	28.6/8.8	30/6.3	514	31%
707	Translationally-controlled tumor protein	*Solanum lycopersicum*	gil75254748	19.0/4.6	22/4.2	317	29%
721	Ribosomal protein L12-1a	*Nicotiana tabacum*	gil20020	20.3/6.3	20/4.3	293	29%
725	homolog to Peptidyl-prolyl cis-trans isomerase (Cyclophilin)	*Solanum lycopersicum*	gil16231886	26.7/9.4	20/7.4	759	46%
754	Peptidyl-prolyl cis-trans isomerase (Cyclophilin)	*Solanum lycopersicum*	gil118103	18.2/8.2	18/8.9	674	68%
755	Glutathione peroxidase	*Solanum lycopersicum*	gil5892354	21.5/9.0	18/8.2	887	65%
788	Constitutive plastid-lipid associated protein	*Solanum lycopersicum*	gil75266239	20.1/8.8	16/4.8	184	26%
807	60S acidic ribosomal protein-like protein	*Solanum tuberosum*	gil76160941	11.5/4.6	15/4.1	635	51%
822	Nucleoside diphosphate kinase	*Solanum lycopersicum*	gil575953	15.5/6.8	13/6.5	457	38%
Protein that decreased in presence of sucrose							
9	homolog to Patellin	*Solanum lycopersicum*	gil16236536	25.6/8.1	115/4.6	426	39%
322	Rubisco activase [d]	*Solanum pennelli*	gil2707330	50.9/8.6	43/5.3	125	45%
380	homolog to Peroxidase	*Solanum lycopersicum*	gil15198373	35.5/6.1	40/8.3	661	39%
639	Carbonic anhydrase	*Solanum lycopersicum*	gil56562177	34.8/6.7	27/6.7	727	50%
784	Ferredoxin (Isotype II)	*Solanum lycopersicum*	gil1589259	10.7/4.1	16/3.8	230	35%
799	Cu-Zn Superoxide dismutase (chloroplast)	*Solanum lycopersicum*	gil134682	15.5/6.8	16/5.4	328	17%

Proteins were extracted from leaf of twenty-day old tomato plantlets grown *in vitro* in presence (3%) or absence (0%) of sucrose. Eleven and five protein presented increase and decrease content, respectively. Analysis was performed with Phoretix Expression software. Spot were analysed using LC-MS/MS QTOF micro. Proteins were identified by MASCOT. All identifications were statistically significant ($p<0.05$). Mr, relative molecular mass; pI, isoelectric point.

[a] Molecular weight and isoelectric point estimated from the two-dimensionnal gel.

[b] MOWSE score calculated by MASCOT.

[c] Pourcentage of protein sequence covered by identified peptide.

[d] Protein identified by SELDI-TOF MS.

4.5.2 Proteins that increased with exogenous sucrose

Among the eleven proteins increasing in abundance in photomixotrophic plantlets, several were involved in protein synthesis and degradation, and nucleotide synthesis. In particular, two ribosomal proteins, a ribosomal protein L12-1a and a 60S acidic ribosomal protein, showed 2.35- and 1.65-fold increases in content in sugar-feed plantlets (Fig. 4.2). Following the same trend, two peptidyl-prolyl cis-trans isomerase (cyclophilin), which participates in protein folding, accumulated 1.4- and 1.9-fold in presence of exogenous sucrose (Fig. 4.2). Moreover, the translationally-controlled tumor protein, involved in translation, presented the highest increased in protein level with a 3.2-fold higher accumulation in presence of exogenous sucrose (Fig. 4.2). In eukaryote, the proteasome is

responsible for the degradation of a large number of proteins and is involved in a broad range of regulatory process. The 20S proteasome alpha 6 subunit was more abundant in sugar-fed plantlets (Fig. 4.2). A protein involved in nucleotide metabolism was also more abundant in sucrose-fed plantlets; i.e. a nucleoside diphosphate kinase (NDPK) (Fig. 4.2). NDPK are responsible for the transfer of a gamma-phosphate from a donor nucleoside triphosphate (NTP) to an acceptor nucleoside diphosphate (NDP).

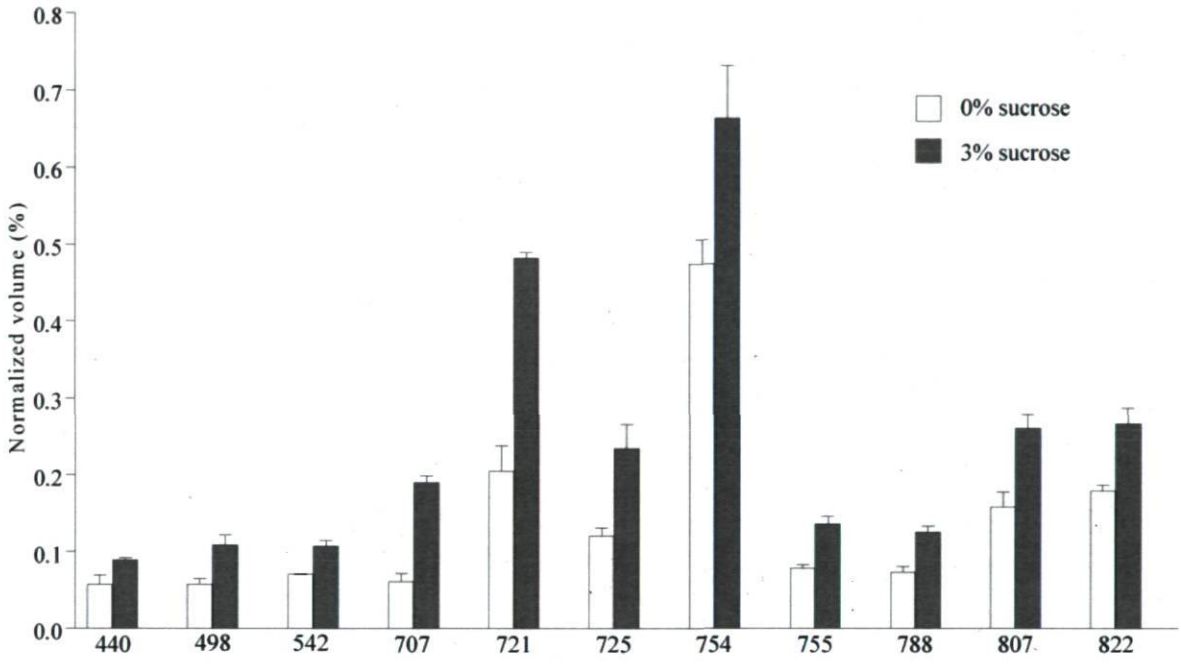

Figure 4.2 Normalized volume (%) of identified proteins showing increased abundance in tomato plantlets cultured *in vitro* under photomixotrophic conditions (3% sucrose). Normalization was performed by Phoretix Expression software by dividing the volume of each spot by the total volume of all the spots on the 2-DE. Data represent the mean normalized volume (%) of four biological replicates ± SE. All spots were statistically significant ($p<0.05$). Spot numbers are presented. Spot protein identification is as follow: 440, 20S proteasome alpha 6 subunit; 498, Porin I ; 542, homolog to NAD-dependent epimerase/dehydratase; 707, Translationally-controlled tumor protein; 721, Ribosomal protein L12-1a; 725, homolog to Peptidyl-prolyl cis-trans isomerase (Cyclophilin); 754, Peptidyl-prolyl cis-trans isomerase (Cyclophilin); 755, Glutathione peroxidase; 788, Constitutive plastid-lipid associated protein; 807, 60S acidic ribosomal protein-like protein; 822, Nucleoside diphosphate kinase.

Additionally, four proteins involved in various reactions and physiological processes were shown to increase in abundance under photomixotrophic conditions. In particular, a voltage-dependent anion channel (porin I) accumulated 1.9-fold (Fig. 4.2). Porins are situated at the outer membrane of mitochondria and functions in the transport of

metabolites between the cytoplasmic and the mitochondrial compartments. A plastid lipid-associated protein (PAP), CHRDC, showed 1.7-fold higher abundance in sucrose-feed plantlet (Fig. 4.2). In leaves, PAPs are nuclear encoded protein localized in chloroplasts and are involved in the sequestration of hydrophobic compounds. A protein homolog to NAD-dependent epimerase/dehydratase followed the same tendency (Fig. 4.2). This protein possesses a NMR1-like and a nucleoside diphosphate sugar epimerase conserved domain, but its function is nebulous. Additionally, a glutathione peroxidase, a class I peroxidase involved in hydrogen peroxide detoxification to H_2O, was 1.7-fold more abundant in sugar-fed plantlet (Fig. 4.2).

4.5.3 Proteins that decreased with exogenous sucrose

In the present study, only very few spots were less abundant in plantlets grown under photomixotrophic conditions (Table 4.1). Five out of seven proteins showing decreased content were identified in plantlets grown on high sucrose. Of these five proteins, three were involved in crucial steps of photosynthesis. The Rubisco activase (RCA), the carbonic anhydrase (CA) and the ferredoxin II (FDII) showed 1.4-, 1.7- and 2.3-fold decreased content when sucrose was added to the MS medium (Fig. 4.3). The Rubisco activase is definitively the spot showing the largest volume on the gels and therefore a 27 % increase in content is certainly noticeable and metabolically relevant. Furthermore, we identified a chloroplast Cu-Zn superoxide dismutase (Cu-ZnSODchl) protein, which was only present in plantlets grown under photoautotrophic conditions (Fig. 4.3). Cu-ZnSODchl is an important enzyme involved in the scavenging of $O_2^{\cdot}$ and detoxification to H_2O_2 in the chloroplast. Additionally, a protein homolog of peroxidases was 2-fold less abundant (Fig. 4.3). Search for conserved domains in this protein revealed the presence of a secretory peroxidase domain. This protein would be member of the class III peroxidase family and therefore could be involved in cell wall metabolism, lignification and suberification, auxin catabolism, and defense. Lastly, a protein homolog to patellin1 from *Cucurbita pepo* (68% identity) and *Arabidopsis* (56% identity) showed the highest fold change in this experiment with 5.6-fold lower content (Fig. 4.3). This protein possesses a sec14p-like lipid-binding domain and therefore is a secreted protein with the ability to transfer phosphatidylinositol (PtdIns). In particular, Sec14p-like proteins participates in membrane trafficking (Peterman *et al.*, 2004).

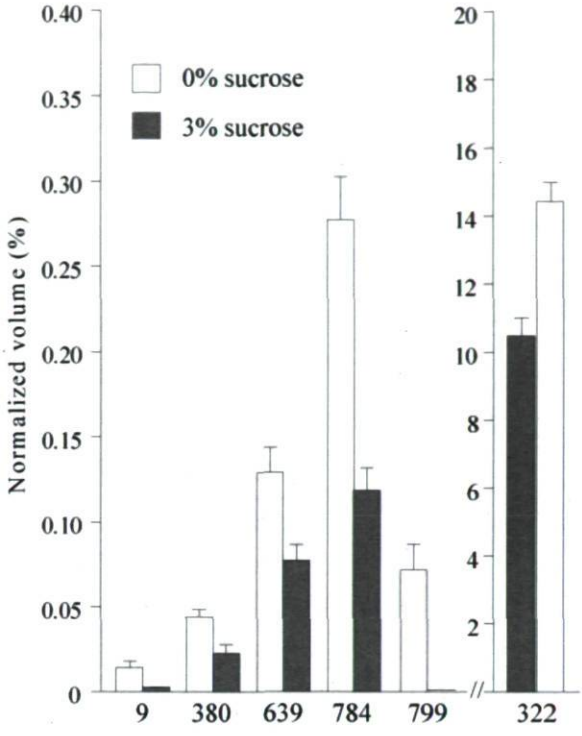

Figure 4.3 Normalized volume (%) of identified proteins showing decreased abundance in tomato plantlets cultured *in vitro* under photomixotrophic conditions (3% sucrose). Normalization was performed by Phoretix Expression software by dividing the volume of each spot by the total volume of all the spots on the 2-DE. Data represent mean normalized volume (%) of four biological replicates (± SE). All spots were statistically significant ($p<0.05$). Spot numbers are presented. Spot protein identification is as follow: 9, homolog to Patellin; 380, homolog to Peroxidase; 639, Carbonic anhydrase; 784, Ferredoxin (Isotype II); 799, Cu-Zn Superoxide dismutase; 322, Rubisco activase.

4.5.4 Photosynthesis

In order to further investigate the impact of exogenous sucrose on tomato plantlet photosynthesis, photosynthetic data were recorded with a portable infrared gas analyzer, LI-6400. Compared to plantlets grown under photoautotrophic conditions, sucrose-feed plantlets showed a 27% lower light saturated photosynthetic rate (A_{max}) (Fig. 4.4A). In parallel, the photosynthetic quantum efficiency (Φ) was lower (-38%) and the light compensation point was higher (+34%) in plantlet grown in presence of sucrose, but they were not statistically significant (Fig. 4.4B and C). Cellular respiration rate did not respond to treatments (Fig. 4.4D).

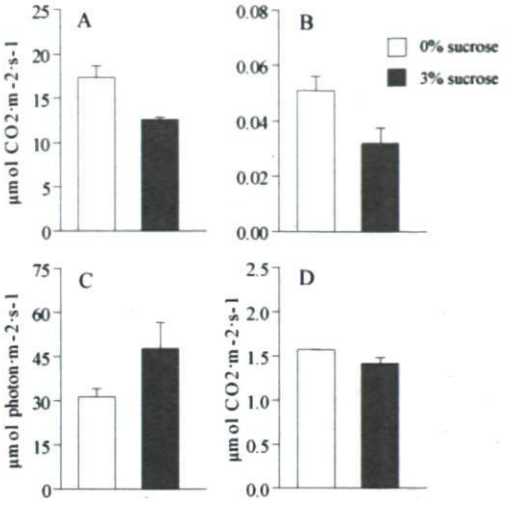

Figure 4.4 Photosynthetic parameters of twenty-day old *in vitro* tomato plantlets grown under photomixotrophic (3% sucrose) and photoautotrophic conditions (0% sucrose). Light saturated maximum (Amax), apparent quantum efficiency (QE), light compensation point (LCP), and apparent cellular respiration (Resp) are shown in (A), (B), (C) and (D) respectively. Leaf net photosynthetic rate was measured for seven different levels of light between 0 and 1000 µmol photons m-2 s-1 at a CO_2 concentration of 400 µmol m^{-2} s^{-1}. A light response curve was established and photosynthetic parameters were calculated with the Photosyn Assistant software. Data represent the mean of three biological replicates (± SE). Amax was statistically significant at $p \leq 0.05$.

4.6 Discussion

Two-dimensional electrophoresis (2-DE) together with mass spectrometry was instrumental in identifying several proteins involved in plantlet's responses to standard micropropagation media containing high sucrose. Proteomic analysis allowed the identification of proteins that play a central role in cell growth, oxidative stress and photosynthesis. Hence, these results provide novel data on the downstream impact of exogenous sucrose on micropropagated plantlet's physiological responses.

Several of the proteins shown to increase under photomixotrophically growth conditions were involved in protein synthesis (translation) and proteolysis. These include two ribosomal proteins, a translationally-controlled tumor protein (TCTP), two cyclophilin type peptidyl-prolyl cis-trans isomerase, and a 20S proteasome alpha 6 subunit. Ribosomes are a complex machinery build-up from numerous proteins. The 60S acidic ribosomal protein-like protein is a close homolog to ribosomal protein LP2 from Arabidopsis. LP2 is involved in the formation of the ribosomal stalk, a domain containing the ribosomal protein

LP0, LP1, LP2, and LP3, presumed to be involved in binding the translation elongation factors to the ribosome. In human, suppression of LP2 mRNAs and proteins reduces the cell growth rate probably by impairing the ability of the 60S ribosomal subunit to assemble with the 40S subunit (Martinez-Azorin *et al.*, 2008). In opposition to the cytoplasmic protein LP2, the protein L12-1a is a nuclear-encoded ribosomal protein present in the 50S subunit of chloroplast (Elhag *et al.*, 1992). Interestingly, while being targeted to a different part of the cell, these two ribosomal proteins show high homology. The chloroplastic L12-1a and eukaryotic LP2 protein are evolutionary-related to the ancestral prokaryote L7/L12 (Bommer & Stahl, 2005). Similarly to its counterparts, the chloroplastic L12-1 protein is a component of the ribosomal stalk and likely involved in translation accuracy, ribosomal translocation during protein synthesis and cellular growth (Kirsebom *et al.*, 1986). Thus, these results suggest that this group of ribosomal protein is particularly responsive to sucrose level. This goes in line with result by Contento et al (2004) in sugar-starved *Arabidopsis* suspension cells, which showed important down-regulation of the transcript level of several ribosomal proteins.

The elongation phase of translation is a crucial step in protein synthesis. One of the proteins that accumulated in presence of sugar, the TCTP, plays a key role in that process. *In vitro* and *in vivo* studies indicated that TCTP interacts with eukaryotic elongation factor, eEF1A and eEF1Bβ, and could act as guanine nucleotide dissociation inhibitor (GDI) (Cans *et al.*, 2003). Elongation factors are involved in translational elongation as they participate in the enzymatic delivery of aminoacyl tRNA to the ribosome following GTP hydrolysis. Functionally, TCTP is presumed to form a complex with eEF1A-GDP, inhibiting the dissociation of bound GDP, and interferes with its recruitment by *de novo*-aminoacylated tRNA. Thus, TCTP may be involved in tRNA direct channeling i.e. efficient translocation of tRNA from its specific aminoacyl-tRNA synthetase to the ribosome and reciprocally, from the ribosome to the aminoacyl-tRNA synthetase, without liberation of tRNA in the cytoplasm. In plant, TCTP expression increases during ripening of strawberry fruits and in cotyledon of *Pharbitis nil* maintained in the dark (Lopez & Franco, 2006). TCTP was also identified in actively proliferating plant tissue, for instance, in embryo-specific protein in *Cyclamen persicum* (Lyngved *et al.*, 2008) and in dividing cell in root caps of *Pisum sativum* (Woo & Hawes, 1997).

Once protein synthesis is terminated, proteins must be folded into their functional three-dimensional structure. In this perspective, two cyclophilin type peptidyl-prolyl cis-trans isomerase increased in abundance under photomixotrophic conditions. Cyclophilins, as members of the immunophilin family of protein, are functionally involved in protein folding by isomerisation of peptide bond adjacent to the proline residues through their peptidyl-prolyl cis-trans isomerase activities. Globally, cyclophilins are involved in a variety of cellular processes and to participate in plant development and stress responses (Romano *et al.*, 2004).

To allow efficient use of available energy and building block, damaged and unneeded proteins are ultimately targeted to the proteasome for their degradation by proteolysis. Previously, the proteasome was shown to be important in the response to various stresses. Specifically, the 20S proteasome alpha 6 subunit expression was reported to be stimulated by jasmonate, salt and salicylic acid (Kim *et al.*, 2003). Alternatively, proteasome activity has also been shown to evolve in the course of plant development. In particular, increased activity has been observed in actively dividing cells. Therefore, the proteasome is generally considered to have a central role in controlling the level of regulatory proteins in these tissues, especially, proteins involved in the cell cycle (Bahrami & Gray, 1999; Kurepa & Smalle, 2008).

All in all, these results suggest that the sugar-fed plantlets show increased protein metabolism possibly through increased ribosomal proteins and cyclophilin content, tRNA direct channeling, and increased protein recycling. Furthermore, these results are coherent with the implication of sugar in the regulation of protein synthesis and cell proliferation (Riou-Khamlichi *et al.*, 2000). Therefore, the proteins profiles of leaves from sugar-fed plantlets suggest that they display a more active cell division.

Other proteins involved in cell growth were responsive to sugar level *in vitro*. Nucleoside diphosphate kinases (NDPK) fulfill important housekeeping function as they are involved in the biosynthesis of nucleic acid by producing NTPs using ATP. In tomato leaf, NDPK was shown to increase following wounding (Harris *et al.*, 1994). Additionally, sequence analysis revealed that this protein is a type I NDPK and 98% homolog to cytosolic NDPK1 from *Solanum chacoense*. Interestingly, a recent study showed that the

NDPK1 protein level increased through the growth phase in *Solanum chacoense* cell culture and that this response was dependent on sucrose availability (Dorion *et al.*, 2006). Moreover, highest NDPK1 abundance was observed in meristematic tissue of potato plants where it was postulated to be mainly involved in the biosynthesis of the precursor of the cell wall matrix. Accordingly, production of UTP is necessary to UDP-sugar synthesis, particularly upon cell division for cell plate formation (Backues *et al.*, 2007; Dorion *et al.*, 2006). This result goes in line with the implication of sugar in the control of cell division.

By opposition, we report the lower abundance of a patellin1 (PAT1) homolog. In *Arabidopsis*, PATL1 was shown to be involved in membrane trafficking, and more particularly, in the development and maturation of the cell plate during late cytokinesis (Peterman *et al.*, 2004). In zucchini, studies support a function in membrane trafficking for CpPATL1, but no direct role was determined (Peterman *et al.*, 2006). Yet, these results are somewhat conflicting with evidence showing increase cell proliferation in plantlets grown under photomixotrophic conditions. Such discrepancy could be attributable to species-specific variation in proteins function. Alternatively, the decrease content of PATL1 in sugar-fed plantlets could be attributable to the perturbation of BR-mediated growth response such as cell elongation. Analysis of brassinosteroid (BR)-treated *Arabidopsis* plants, and BR-deficient and BR-insensitive mutants revealed an important role of PATL1, -2 and -4 in BR-mediated response (Deng *et al.*, 2007). The absence study of mutant with lost of function renders difficult the assessment of patellin precise role. Hence, the exact meaning of the lower abundance of PATL1 protein remains obscure.

Mitochondrial metabolism was also affected by exogenous sucrose. Mitochondrion functions rely on continual flow of metabolites from the cytoplasm and specific transporters are needed to facilitate the exchanges between the two compartments. Accordingly, voltage-dependent anion channels (VDAC) were previously shown to be involved in the translocation of negatively charged metabolites through the outer mitochondrial membrane (Heins *et al.*, 1994). Additionally, VDAC were also demonstrated to be successfully translocate DNA in mammals. Recently published data indicate that VDAC (porin I) from potato have an important role in tRNA transport into mitochondria (Salinas *et al.*, 2006). Hence, the increase content of porin I in plantlets grown in presence

of exogenous sucrose may suggest an increased demand for metabolites and/or tRNA for translation and biogenesis in mitochondria.

Photosynthesis has been the bottom-line for many researchers interested to understand the causes of acclimatization problems of micropropagated plantlets. Proteomic analysis of tomato plantlets under contrasting sucrose levels revealed important perturbation of specific proteins involved in photosynthesis. For example, in the photosynthetic electron transport chain, ferredoxin is an electron carrier involved in the production of reducing power (NADPH) essential to various processes in plant such as nitrogen and sulphur assimilation, secondary metabolism and CO_2 assimilation. *Solanum tuberosum* antisense lines of leaf-specific ferredoxin I (FDI) with reduced level up to 40% of wild type showed several physiological alterations such as increase cyclic electron flow and decrease CO_2 assimilation rates (Holtgrefe *et al.*, 2003). Interestingly, upon transfer from tissue culture media to soil, some antisense lines, most likely with FDI content below 40% of wild type, did not acclimatized to autotrophic conditions. Sugar inhibition of photosynthesis *in vitro* may, in part, result from the alteration of electron flow toward production of NADPH by FD. These results suggest that reduction of FDII in sugar-fed plantlets to 43% of photoautotrophically grown plantlet's content could limit acclimatization under higher light regime by reducing electron flow and generating excess electron pressure. Under situation of excess, electron are managed sequentially by the malate valve, cyclic electron flow and ultimately lead to production of toxic $O_2^{\cdot-}$. Additionally, exogenous sugar negatively impacts the abundance of a chloroplast-localized β-isoform carbonic anhydrase (CA). This enzyme was proposed to enhance CO_2 availability in the chloroplast stroma by the interconversion of CO_2 and HCO_3^- (Moroney *et al.*, 2001). In *Nicotiana tabacum*, CA antisense mutants with 1% of wild-type activity, had only a small decrease in photosynthesis and showed no visible change in phenotype (Price *et al.*, 1994). Interestingly, the study of *Arabidopsis* knockout and antisense mutants of chloroplast targeted CA1 gene led to important mortality of seedling at the transition to photoautotrophic growth (Ferreira *et al.*, 2008). In their experiment, these authors showed that, in transgenic seedlings that survived, the impact of low CA activity on photosynthesis and growth was almost absent when true leaves were formed, suggesting that mechanisms of compensation were initiated later in development. These results suggest that 41% lower

CA content in sugar-fed plantlets may have some impact on early transition to photoautotrophic growth following transfer *ex vitro*. The results also show that exogenous sucrose lowered Rubisco activase abundance. The Rubisco activase (RCA) is a chaperone-like enzyme necessary to activate and maintain Rubisco activity by releasing the inhibitory sugar phosphate, mainly RuBP (Zhang *et al.*, 2001). Reduce expression of Rubisco activase to as much as 5% of wild type level had no impact on photosynthesis and only severe reduction in RCA abundance leads to decrease CO_2 assimilation (Hammond *et al.*, 1998; Mate *et al.*, 1996). Therefore, high RCA content is not necessary for sustained photosynthesis under steady-state photosynthetic conditions (Mott & Woodrow, 2000). Although RCA may have limited impact on photosynthesis, the decrease of RCA content in combination with FDII and CA could act synergistically on photosynthesis and acclimatization success of sugar-feed plantlets. These data corroborates with the lower light saturated photosynthesis (A_{max}) and quantum efficiency (Φ) in sugar-fed tomato plantlets. Changes in abundance of these photosynthetic proteins suggest that plantlets respond to high exogenous sugar by altering the electron flow through the chloroplastic transport chain, CO_2 availability and RCA content.

A chloroplast-localized plastid-lipid associated protein (PAP), CHRDC, also referred as plastogloblin, with little known function was identified to be more abundant in sugar-fed plantlets. Plastoglobules are plastid-localized lipoprotein particles serving in lipid biosynthesis and storage subcompartement of thylakoid membranes (Austin *et al.*, 2006). Plastoglobulin, as structural proteins of plastoglobule, have been shown to be extremely responsive to several biotic and abiotic stresses such as reactive oxygen species, wounding, drought and high light (Brehelin *et al.*, 2007). In particular, expression of the tomato CHRDC gene was identified to be present in several tissues and stable under heat stress (Leitner-Dagan *et al.*, 2006).

Proteins involved in the production and processing of hydrogen peroxide (H_2O_2) in the chloroplast were identified to accumulate to different extent in presence of sucrose. Reactive oxygen species (ROS) are constantly produced in chloroplasts, peroxisomes and mitochondria. In plants, toxic ROS are scavenged by different antioxidants or by enzymatic reactions. Superoxide dismutases (SOD) represent the first line of defense against ROS.

Afterwards, the ascorbate-glutathione and the glutathione peroxidase cycle detoxify H_2O_2 to water. Sugar-feeding had a negative impact on accumulation of a chloroplastic Cu/ZnSOD (Cu-ZnSODchl) in *in vitro* tomato plantlets. Previous studies have reported the down-regulation of a Cu-ZnSODchl gene in *in vitro* tomato plantlets (Dubuc, 2010) and a decrease Cu-ZnSODchl activity in *Trifolium repens* explants (Slesak *et al.*, 2006) in medium supplemented with sugar. Recently, sucrose was identified to negatively regulate the activity of *Arabidopsis* chloroplastic Cu/ZnSOD, CSD2, via miRNA-directed mRNA cleavage by the sugar regulated miR398 (Dugas & Bartel, 2008). Therefore, reduced Cu-ZnSODchl expression and activity have been suggested to be associated to the inhibitory effect of carbohydrate on photosynthesis. Accordingly, there seem to be a relationship between Cu/ZnSODchl and photosynthetic rate. Inversely, a glutathione peroxidase (GPX) accumulated to higher level in sugar-fed plantlets. GPX enzymes are present in several subcellular compartments in plant cells. Sequence analysis using iPSORT (Bannai *et al.*, 2002) revealed that GPX possess a chloroplast transit peptide. Moreover, tomato GPX shares 79% identity (87% similarity) with chloroplast targeted AtGPX1 from *Arabidopsis thaliana*. AtGPX1 was shown to be induced in response to heat and salt stresses (Milla *et al.*, 2003). An earlier study in our lab demonstrated an up-regulation of a chloroplastic glutathione reductase mRNA in sugar-fed tomato plantlets (Dubuc, 2010). Glutathione reductase is responsible for the regeneration of reduced glutathione, an important antioxidant. Sugar has proven to enhance plant defense (Couée *et al.*, 2006) and increase GPX content could be part of the arsenal of sugar-based tolerance to stress. Therefore, sugar treatment could enhance antioxidant capacity of *in vitro* plantlets and increase their capacity to cope with the *ex vitro* harsh environment. Additionally, increase GPX may be associated to lower FDII content and consequently serve to scavenge reduced O_2 produced by the excess electron from the electron transport chain content. However, questions must be addressed regarding the opposite trend followed by GPX and Cu-ZnSODchl abundance in *in vitro* plantlets.

The cellular level of H_2O_2 determines plant growth and development. For instance, class III peroxidases (POX) regulate cell wall properties by the generation of ROS using H_2O_2 (hydroxylic cycle) or by the reduction of H_2O_2 (peroxidative cycle). Indeed, the hydroxylic cycle produce ROS which attack the cell wall matrix, thus favoring cell wall

loosening and elongation. Inversely, the peroxidative cycle enhance cross-linking of cell wall and biosynthesis of lignin (Passardi *et al.*, 2004). Besides, many studies have recognized the importance of peroxidases in plant defense against biotic and abiotic stress because of their capacity to increase cell wall rigidity and to raise ROS production (Hiraga *et al.*, 2001). Although, any assumption concerning the lower class III POX abundance are precarious because of the diversity of function and the paucity of data on the role of individual POX, this result could have important role in plantlet physiology and biochemistry and a major implication in plantlet's capacity to confront stresses during acclimatization.

Proteomic and photosynthetic results corroborate with the sugar repression of photosynthesis in *in vitro* photomixotrophic plantlets. Furthermore, we report the increase abundance of protein that functions in protein elongation, folding and degradation in cytoplasm, mitochondria and chloroplast. Data presented in this study agrees with earlier large scale transcriptomics analyses (Osuna *et al.*, 2007; Price *et al.*, 2004; Thimm *et al.*, 2004). Previous data on the impact of sucrose on gene expression suggested an important role of sucrose on the inhibition of cell expansion (Dubuc, 2010). This new study strongly suggests that in presence of exogenous sucrose, plantlets exhibit high rate of cell division and therefore high mitotic activity. From this, we can hypothesise that sucrose would artificially alter leaf development and maintain tissue under active division. Sugar-fed tomato plantlets display similar characteristics than those observed during the transition from heterotrophy to autotrophy of leaves. For instance, photosynthesis, and secondary wall synthesis are inhibited and ribosomal proteins, and protein synthesis are stimulated as observed in young leaves of *Populus tremula* (Sjodin *et al.*, 2008). The coordination of cell division and expansion has been a matter of debate for the last decades. Studies have demonstrated that both process are highly coordinated and that a strong relationship exist between cell number and final leaf area (Cookson *et al.*, 2005; Granier *et al.*, 2000). On the one side, plantlets grown under photomixotrophic conditions are photosynthetically less competent and show a modified ROS scavenging system; on the other side, the presence of exogenous sucrose could possibly allow greater plasticity in leaf development and final leaf area. Although, the presence of sucrose in *in vitro* culture medium may be beneficial, to some extend, for plantlets adaptation to *ex vitro* conditions, the establishment of a short

acclimatization phase *in vitro*, which would consist in lowering sucrose concentration before transfer *ex vitro*, could allow the correction of abnormalities induce by exogenous sucrose.

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Chapitre 5 : Conclusion

Les plantes cultivées *in vitro* développent un phénotype unique qui résulte des conditions particulières qui prévalent dans les contenants de culture. La culture *in vitro* offre un environnement confiné, complètement différent des conditions naturelles tant au niveau de l'atmosphère, de la composition du milieu que de l'absence d'agents pathogènes. Ces différences majeures entraînent des complications lors du transfert des plantules vers les conditions *ex vitro*. Selon l'espèce et les conditions de culture, un pourcentage variable des plantules cultivées *in vitro* ne survivent pas la transition vers l'environnement extérieur incontrôlé. Des études antérieures ont rapporté que les conditions de culture *in vitro* avaient des impacts néfastes sur la physiologie, l'anatomie et la morphologie des plantules (Pospisilova *et al.*, 1999). Plus particulièrement, on sait que le saccharose exogène joue un rôle majeur dans la régulation de la photosynthèse et la survie des plantules *ex vitro* (Hdider & Desjardins, 1994; Le *et al.*, 2001). On sait par ailleurs que la plante utilise la teneur intracellulaire en hydrates de carbone pour réguler plusieurs processus physiologiques et biochimiques clés nécessaires à leur métabolisme harmonieux. L'apport de saccharose exogène en culture *in vitro* interfère donc potentiellement avec les mécanismes de régulation de croissance des plantules. Nous pensons qu'il est en grande partie responsable des aberrations physiologiques et morphologiques induites par la culture *in vitro*. C'est dans ce contexte particulier que s'est inscrit la présente thèse. Pour étudier l'effet du saccharose sur la physiologie des plantules *in vitro*, nous avons opté pour l'utilisation des outils performants qu'offre aujourd'hui la génomique. Nos travaux inédits montrent la puissance de la transcriptomique et de la protéomique pour l'étude de la régulation de croissance et les réponses fines des plantules à un environnement stressant et perturbateur.

Le présent projet de recherche avait pour objectif principal d'apporter de nouvelles données sur l'impact des conditions de culture *in vitro* sur le métabolisme des plantules de tomate (*Solanum lycopersicum)* et sur leur acclimatation *ex vitro*. La tomate était un choix tout indiqué pour cette étude. En effet, la tomate est une espèce modèle en recherche fondamentale dans le domaine des relations source-puit (Sinha et al., 2002) ainsi qu'en culture *in vitro* (El-Bakry, 2002; Kubota *et al.*, 2001; Le *et al.*, 2001). Une bonne connaissance des métabolismes carbonés et azotés (Devaux *et al.*, 2003; Wang *et al.*, 2001), le grand nombre de gènes et de protéines disponibles dans les banques génétiques et le génome relativement restreint composé d'environ 35 000 gènes sont des facteurs qui ont

justifié l'emploi de cette espèce comme modèle dans ce projet (Van der Hoeven *et al.*, 2002). L'utilisation de ce modèle a permis de révéler un nombre impressionnant de données pertinentes et a accru notre compréhension du phénomène de l'acclimatation des plantules lors du passage des conditions *in vitro* à celles *ex vitro*. Bien que la tomate ne présente pas de problèmes majeurs de survie à l'acclimatation, les résultats obtenus avec cette espèce ont permis d'améliorer les connaissances, ce qui pourrait contribuer à faciliter l'acclimatation d'espèces réellement problématique. En effet, des travaux sont toujours en cours afin d'améliorer l'acclimatation de plusieurs espèces micropropagées telles que la canne à sucre (*Saccharum*) (Rodriguez *et al.*, 2008; Rodriguez *et al.*, 2003), le cocotier (*Cocos nucifera*) (Fuentes *et al.*, 2005; Ledo *et al.*, 2007) et le raisin (*Vitis vinifera*) (Thomas & Ravindra, 2002). La disponibilité grandissante des données génétiques et d'outils de génomique chez des espèces mineures permettra d'améliorer la compréhension des problèmes d'acclimatation et de survie des plantules lors du transfert *ex vitro*.

Ce projet de doctorat comportait trois hypothèses et trois objectifs principaux. Dans le cadre du premier objectif, il s'agissait de montrer que les conditions de culture *in vitro* ont un effet marqué sur l'expression des gènes associés à la photosynthèse, à la biosynthèse des sucres et des protéines. En d'autres termes, nous nous intéressions à comparer le profil d'expression des plantules *in vitro* à celui de plantules transférées en conditions non contrôlées *ex vitro*. Le second objectif consistait à évaluer l'effet à court terme du retrait du saccharose du milieu de culture sur l'expression des gènes afin de mieux caractériser l'impact de son absence lors du transfert *ex vitro*. Dans un troisième temps, nous voulions observer l'impact du saccharose exogène sur la synthèse et l'accumulation des protéines suite à un cycle de culture en conditions *in vitro*.

Pour atteindre le premier objectif, nous avons développé, dans notre laboratoire, une biopuce à ADN de la tomate comportant 122 gènes sélectionnés aux fins de l'étude. Les gènes déposés sur la biopuce à ADN étaient impliqués dans une diversité de fonctions métaboliques comme le métabolisme énergétique, carboné et azoté, la croissance et le développement, la défense, la synthèse et la dégradation protéique, les fonctions cellulaires, la transcription et la transduction de signaux.

Les résultats obtenus dans le cadre de cet objectif ont permis de montrer que le profil d'expression des plantules *in vitro* diffère grandement de celui des plantules acclimatées. Il semble que l'environnement *in vitro* entraîne une surexpression de gènes impliqués dans la photosynthèse et l'assimilation de l'azote et une sous expression des gènes impliqués dans le métabolisme énergétique et carboné. De plus, nous avons obtenus des résultats très intéressants et originaux sur la régulation du système de défense chez les plantules *in vitro*. Notamment, les plantules *in vitro* ont surexprimé certains gènes associés à la voie de signalement de l'acide salicylique et sous exprimé des gènes associés à la voie de signalement de l'acide jasmonique. En somme, les résultats obtenus suggèrent que les conditions *in vitro* ont un effet négatif sur la force des puits et favorisent l'assimilation de l'azote. Pour étudier plus en détail la réponse de défense observée chez les plantules, des données supplémentaires ont été générées par PCR en temps réel et ont permis de montrer que les conditions *in vitro* causent une perturbation du métabolisme des espèces réactives de l'oxygène (ROS), et ce, de façon spécifique à chaque compartiment de la cellule. Globalement, les données tendent à montrer que le sucre exogène aurait un rôle important dans la réponse génique liée au métabolisme des ROS, de la photosynthèse, de l'assimilation du nitrate et du système de défense.

A priori, on s'attendait à ce que les conditions de culture *in vitro* provoquent une sous expression des gènes impliqués dans la photosynthèse chez les plantules *in vitro*. Cependant, contrairement à l'effet négatif du saccharose sur l'expression des gènes impliqués dans la photosynthèse chez les plantes témoins poussant en conditions normales et que plusieurs auteurs ont décrit (Koch, 1996), nos résultats ont plutôt montré que les conditions limitant la photosynthèse *in vitro* ont engendré une surexpression des sous-unités du photosystème I et II et de la Rubisco activase. Ces résultats seraient possiblement attribuables aux faibles conditions de lumière *in vitro*. De plus, contrairement à nos attentes, nous avons observé une inhibition globale du métabolisme carboné probablement attribuable à un engorgement des puits radiculaires. Parallèlement, notre hypothèse prévoyait que les gènes associés à la défense seraient stimulés par les conditions de culture *in vitro*. Nous avons plutôt observé une ségrégation de la réaction de cette classe de gènes en deux groupes distincts. À cet égard, les gènes impliqués dans le sentier de l'acide salicylique ont été surexprimés alors que ceux associés au sentier de l'acide jasmonique ont

été sous exprimés. Finalement, les plantules affichent une réponse génique importante au niveau du métabolisme azoté probablement attribuable à la concentration élevée en azote que l'on observe dans le milieu de culture MS et qui est typiquement 80 fois supérieures à celle qu'y est présente dans le sol. Plus précisément, nous avons observé que les gènes impliqués dans l'assimilation de l'azote, à l'exception du transporteur de nitrate NRT1.1, étaient surexprimés. D'une part, cette réponse pourrait servir à assimiler la très forte concentration d'azote, vraisemblablement supérieure au besoin des plantules, présente dans le milieu MS. D'autre part, la sous expression de NRT1.1 chez les plantules *in vitro* pourrait servir à limiter l'assimilation des nitrates, présents en concentrations élevées dans le milieu MS, en fonction des besoins en azote restreint par la faible force des puits.

Le système de caractérisation de l'expression génique employée dans cette étude comportait certaines limitations qui nous empêchent toutefois de généraliser les conclusions. Malgré le fait que nous ayons prélevé la deuxième feuille des plantules *in vitro* et *ex vitro* pour l'étude, nous sommes conscients que les feuilles n'avaient possiblement pas le même stade de développement. Ainsi, une différence dans le développement des feuilles peut avoir influé sur la réponse génique des plantules. Cependant, la comparaison de la deuxième feuille des plantules *in vitro* et des plantules *ex vitro* s'est avérée la meilleure option pour étudier les changements d'expression génique qui surviennent au cours de l'acclimatation et pour identifier des gènes candidats pouvant expliquer les problèmes d'acclimatation. Par ailleurs, au début de nos travaux, il n'existait aucune biopuce à ADN à large spectre pour la tomate. Pour développer notre propre biopuce à ADN, nous avons donc sélectionné plus de 120 gènes pour lesquels nous avons produit des amplicons de longueur variant entre 200 et 800 paires de bases. Par conséquent, la sélection *a priori* des gènes à introduire sur la biopuce constitue en elle même un biais sur la nature des résultats que nous pouvions obtenir. Ainsi, compte tenu des gènes que l'on a choisis pour mettre sur la puce, on pouvait s'attendre à obtenir des résultats sur la régulation des gènes photosynthétiques puisqu'ils ont été délibérément inclus sur la biopuce. Néanmoins, nous étions conscients des limites de notre dispositif et désireux d'obtenir de l'information sur des gènes impliqués dans des branches métaboliques spécifiques puisque celle-ci n'était pas disponible ailleurs (Tableau 2.1).

Les résultats de cette première expérience soulèvent certains questionnements qui devraient être étudiés plus en détails. Ne serait-il pas justifié de soumettre les plantules *in vitro* à une étape de pré-acclimatation *in vitro*? Au cours d'un processus de pré-acclimatation *in vitro*, il serait intéressant d'étudier l'impact de l'augmentation de la luminosité et d'une diminution de la concentration en saccharose sur la performance *ex vitro* des plantules dans la perspective d'accroître les compétences photosynthétiques, de rétablir la relation source-puits et de restreindre le stress oxydatif lors du transfert *ex vitro*. Pour pouvoir y arriver de manière pratique, on devra probablement cultiver les plantules sur milieu liquide afin de faciliter le changement de milieu de culture et ainsi préconditionner les plantules au transfert *ex vitro*. De surcroît, il serait pertinent d'étudier l'impact de la modification de l'expression des gènes impliqués dans l'atténuation des ROS au niveau des mitochondries et des chloroplastes sur la survie des plantules lors de l'acclimatation. Il faut probablement remettre en question et mieux définir le rôle des ROS dans le processus d'acclimatation et préciser l'impact du statut oxydatif des plantules *in vitro* lors du transfert aux conditions *ex vitro*. En effet, nous avons montré que l'expression de la Cu/Zn superoxide dismutase chloroplastique était réduite *in vitro* ce qui suggère que les plantules ont une capacité antioxidante restreinte dans le chloroplaste. Ainsi, l'augmentation rapide de la luminosité et de la photosynthèse lors de la transition vers les conditions *ex vitro* pourrait entraîner un stress oxydatif dommageable ou mortel aux plantules *in vitro*. Par ailleurs, les sucres jouent un double rôle dans le métabolisme des ROS. D'une part, ils régulent plusieurs processus responsables de la production de ROS et d'autre part, ils sont impliqués dans les mécanismes antioxydants (Couée *et al.*, 2006). Dans notre expérience, nous avons observé la surexpression de la glutathion réductase et de l'ascorbate peroxidase, deux gènes codant pour des enzymes impliquées dans le métabolisme des ROS, exprimé dans le chloroplaste. Conséquemment, il sera intéressant de déterminer si le sucre a un effet protecteur ou plutôt un impact négatif lors du passage des plantules des conditions photomixotrophes à photoautotrophes. En dernier lieu, il serait pertinent de comparer le profil d'expression des plantules *in vitro* mises en croissance en conditions photoautotrophes et des plantes *ex vitro* issues de ces mêmes conditions *in vitro* à plusieurs temps au cours de l'acclimatation. Cette étude permettrait d'apporter des données nécessaires à la caractérisation de la réponse transcriptionnelle des plantules tout

au long de l'acclimatation et de mieux qualifier les avantages et les désavantages des systèmes de culture photomixotrophe et photoautotrophe. De plus, la comparaison du patron d'expression génique des plantules *ex vitro* issues des deux systèmes de cultures permettrait de mieux expliquer les processus d'acclimatation initiés selon les conditions *in vitro* utilisées. Ce dispositif expérimental permettrait de comparer les comportements adaptatifs des plantules photomixotrophes et photoautotrophes lors de l'acclimatation.

Les conclusions du premier chapitre, à l'effet que les plantules *in vitro* affichent une modulation majeure des gènes impliqués dans le métabolisme des ROS, la photosynthèse, l'assimilation de l'azote et la défense, font ressortir une participation importante des sucres lors du transfert *ex vitro*. Cette réponse qui correspond à l'intégration de toutes les réactions d'expression sur une période de 12 jours, nous informe sur les grands patrons d'expression et les différences qui existent au niveau du développement des plantules *in* et *ex vitro*. Pour approfondir notre compréhension des réactions morphogéniques et biochimiques induites par la présence de sucre, nous avons évalué l'effet du retrait du saccharose du milieu de culture sur l'expression des gènes à court terme. Deux groupes de chercheurs se sont attardés à étudier l'effet du retrait du saccharose (Contento *et al.*, 2004) et de l'ajout de glucose (Price *et al.*, 2004) sur le profil d'expression génique de cellules en culture. Contento *et al.* (2004) ont observés qu'après 24h, des protéases vacuolaires impliquées dans l'autophagie étaient induites. Après 48h, plusieurs facteurs de transcription et plusieurs gènes impliqués dans la transduction de signaux, dans la remobilisation des réserves, et dans les mécanismes de défense ont été surexprimées alors que les gènes impliqués dans la division cellulaire, la glycolyse, la synthèse de nucléotide, la synthèse d'acides aminés et de lipide, la transcription et la traduction étaient sous-exprimées. Price et al. (2004) ont rapporté que plusieurs gènes impliqués dans le métabolisme carboné, la transduction de signaux, le transport, et le stress étaient régulés. Ces auteurs ont aussi montré que les gènes impliqués dans la biosynthèse et la transduction de signaux de l'éthylène étaient plus faiblement exprimés en présence de sucre exogène faisant ressortir un lien étroit entre le sucre et la voie signalétique de l'éthylène. Par ailleurs, d'autres chercheurs ont observé l'effet de l'induction d'une carence en sucre grâce à une prolongation de la durée de la nuit de 6 h chez des plantules d'*Arabidopsis* en terre (Thimm *et al.*, 2004). D'une part, Thimm *et al.* (2004) ont montré que l'augmentation de la durée de

la nuit entraînait la sous-expression de plusieurs gènes impliqués dans la photosynthèse, l'assimilation et le transport du nitrate et du sulfate, la synthèse d'acides aminés, de nucléotides, de lipides, de protéines et d'ARN, la division cellulaire, la modification et la synthèse de paroi cellulaire. D'autre part, des gènes impliqués dans le catabolisme des acides aminés, des nucléotides, des lipides, la dégradation de la paroi cellulaire, la biosynthèse et la voie de signalement de l'éthylène et de l'acide abscissique étaient surexprimés. Tous ces résultats, quoique pertinents, sont peu applicables dans un contexte de micropropagation. D'une part, les cellules en culture ne présentent pas l'architecture et le système de communication complexe des plantes, et d'autre part, la culture en terre ne permet pas de mesurer l'effet du confinement *in vitro* sur le retrait du saccharose. Pour mieux définir la contribution du sucre au cours du processus d'acclimatation, nous avons soumis des plantules de tomates ayant séjourné 20 jours sur un milieu contenant du saccharose *in vitro* à un épisode de carence en sucre pendant 24 h *in vitro*. Ce dispositif expérimental fut choisi afin de caractériser l'effet du retrait du saccharose lors du transfert *ex vitro*.

Peu de temps après la production de la biopuce d'ADN de la tomate dans notre laboratoire, de nouveaux outils de transcriptomiques permettant la mesure de l'expression de plusieurs milliers de gènes chez la tomate ont été commercialisés. En particulier, nous avons eu accès à une biopuce à oligonucléotide (TOM2) développé par le « Center for Gene Expression Profiling (CGEP) » du Boyce Thompson Institute. Il s'agissait d'une biopuce utilisant la même plate-forme que celle employée dans le cadre de notre première expérience, disponible à un coût raisonnable et permettant la mesure de l'expression de plus de 11800 transcrits (unigène). La « GeneChip Tomato Genome Arrays » de la compagnie Affymetrix était la seule autre biopuce à large spectre disponible pour la tomate, cependant elle s'avérait une option beaucoup plus dispendieuse. De ces gènes, l'analyse statistique a permis de faire ressortir qu'un peu plus de 250 gènes réagissaient au retrait du sucre en aussi peu que 24 heures. Ces gènes se répartissaient dans un grand nombre de sentiers métaboliques liés aussi bien au métabolisme primaire, qu'à celui des réactions de défense ou de morphogenèse. L'utilisation du logiciel MapMan (Thimm *et al.*, 2004) nous a permis d'effectuer une première classification des gènes régulés en fonction de leur rôle respectif. Quoiqu'essentiel, MapMan ne permettait pas de tenir compte des multiples fonctions que

chaque gène peut occuper dans le métabolisme de la plante. Ainsi, l'assignation des gènes à une classe limitait l'interprétation des résultats. Une étude plus approfondie des résultats nous a permis d'expliquer plus précisément les événements transcriptionnels survenant 24 h après le retrait du saccharose. Les données de la biopuce à ADN ont montré que le transcriptome réagit rapidement aux nouvelles conditions. Plusieurs facteurs de transcription et différentes protéines kinases sont induits, ce qui signifie que le retrait du saccharose enclenche une importante réponse transcriptionnelle et probablement signalétique. Étonnamment, les gènes impliqués dans le catabolisme des réserves, dans la stimulation de la photosynthèse et dans l'arrêt de la croissance cellulaire, normalement activés en situation de carence en sucre, n'ont pas réagit au retrait du saccharose. Seulement deux gènes, l'anhydrase carbonique et une petite sous-unité de la Rubisco, ont été surexprimés en réponse au retrait du saccharose. Ces résultats suggèrent que l'activation des gènes photosynthétiques ne se produit pas à court terme, du moins pas après 24 h. Ainsi, il est possible que les plantules de tomate aient une réponse photosynthétique plus prononcée avant ou après la période de 24 h étudiée dans cette expérience. En revanche, les résultats obtenus montrent que le retrait du saccharose occasionne des modifications majeures quant à l'expression des gènes associés à la paroi cellulaire et à la membrane plasmique. Dans cette expérience, un total de 20 gènes ont affiché un ratio d'expression plus grand que 3 ou plus petit que -3. Les gènes associés à la paroi et à la membrane cellulaire représentaient près de 50% de ces gènes. Plus précisément, nous avons observé la surexpression de gènes impliqués dans la réorganisation et dans la structure de la paroi cellulaire et dans la synthèse des sucres nucléotidiques requis pour la synthèse de la paroi cellulaire ainsi qu'une modification de la nature de la membrane cellulaire. Nos résultats montrent que les sucres régulent activement la voie signalétique de l'éthylène; ces résultats concordent avec ceux d'autres auteurs (Yanagisawa *et al.*, 2003; Zhou *et al.*, 1998) qui observent que le sucre inhibe la biosynthèse de l'éthylène et interfère avec la cascade signalétique induite par ce régulateur de croissance. Globalement, nos résultats indiquent que le saccharose exogène inhibe la biosynthèse de l'éthylène et que le retrait du saccharose du milieu de culture initie une cascade signalétique liée à l'éthylène, ce qui provoque une modification des propriétés pariétales. En lien avec l'éthylène, nous avons noté que plusieurs protéines de défense étaient surexprimées, comme l'on déjà rapporté plusieurs

auteurs (Hermsmeier *et al.*, 2001; Van Zhong & Burns, 2003). En fait, un grand nombre de gènes, régulés à la baisse ou à la hausse, étaient impliqués dans les réactions de défense et de stress chez les plantes. Nous avons également observé que le retrait du sucre du milieu *in vitro* inhibait la biosynthèse de lignine, des cires épicuticulaires, du transport des sucres et de la respiration. Cette réponse n'est pas sans conséquence sur la capacité d'adaptation des plantules aux conditions *ex vitro*. En effet, une diminution de la synthèse de lignine et de cires en réponse à un épisode de carence en sucre pourrait accroître la transpiration et mener à une dessiccation des feuilles et nuire au maintien de la plante. À l'inverse, des gènes impliqués dans la synthèse et la dégradation des protéines et l'autophagie étaient surexprimés, ce qui suggère que la carence en sucre entraîne la dégradation de certaines protéines et, parallèlement, la synthèse *de novo* de protéines impliquées dans la réponse à ce stress environnemental. Le retrait du saccharose cause aussi d'importants réajustements métaboliques notamment au niveau de la régulation du métabolisme du tréhalose et de celui du GABA (Fait *et al.*, 2008; Rolland *et al.*, 2006). D'une part, la surexpression du métabolisme du tréhalose favoriserait l'utilisation des réserves carbonés au détriment de leur entreposage (Contento *et al.*, 2004). D'autre part, nos résultats suggèrent que le métabolisme du GABA pourrait permettre d'assurer un réapprovisionnement du cycle de Krebs en réponse au retrait du saccharose.

Il semble assez clair que le retrait du sucre entraîne une stimulation de la voie de l'éthylène (Price *et al.*, 2004). Cependant, les résultats d'expressions ne nous permettent pas de définir précisément à quoi peut servir cette réponse. De prime abord, certaines données nous suggèrent que l'éthylène induit l'élongation cellulaire. Toutefois, les phénomènes physiologiques comme la sénescence, la réponse au stress et la défense, et le syndrome d'évitement de l'ombre ne peuvent être écartés. Il serait donc essentiel d'étudier les phénomènes physiologiques associés à la stimulation de la voie de l'éthylène observés chez les plantules soumises au retrait du saccharose. Dans cet ordre d'idée, l'utilisation d'outils tels que le mutant « Never-ripe » de la tomate (Lanahan *et al.*, 1994), insensible à l'éthylène, permettrait de préciser l'importance de l'éthylène dans le succès à l'acclimatation et l'interaction entre les sucres et ce régulateur de croissance. Par ailleurs, il serait envisageable d'effectuer des mesures d'expansion et de division cellulaire afin d'observer si le retrait du saccharose a un impact sur ces deux paramètres cellulaires. Des

mesures journalières d'expansion foliaire au cours de l'acclimatation *ex vitro* de plantules issues de conditions de culture *in vitro*, avec ou sans saccharose, sont à considérer pour étudier cette réponse et son implication dans la réponse adaptative des plantules. Advenant une confirmation de l'hypothèse sur la stimulation de l'expansion, il serait alors intéressant de mesurer l'implication de cette réponse cellulaire sur le succès à l'acclimatation. Dans un autre ordre d'idée, des mesures d'expression échelonnée dans le temps après le retrait du sucre permettraient certainement d'observer la réponse physiologique et les cascades transcriptionnelles qui surviennent avant et après 24 h.

Le dernier objectif de cette thèse visait la quantification et l'identification des variations de l'abondance des protéines foliaires des plantules de tomate *in vitro* en présence et en absence de saccharose. Le but de cette expérience consistait à observer l'impact du saccharose exogène sur la synthèse et l'accumulation des protéines suite à un cycle de culture en conditions *in vitro*. À ce jour, aucune étude n'a investigué la variation de l'abondance en protéines de plantules associées à la présence de saccharose exogène au cours d'une longue période. Ainsi, une étude du protéome de la deuxième feuille de plantules de tomate mis en culture *in vitro* en présence et en absence de saccharose pendant 20 jours, a été effectuée à l'aide de la technique de l'électrophorèse bidimensionnelle. Plusieurs protéines affichant une abondance variable ont été identifiées par la suite par spectrométrie de masse. Des mesures d'activités photosynthétiques ont aussi été effectuées afin de mieux comprendre la réponse d'adaptation des plantules à la présence de saccharose exogène.

Les résultats obtenues par l'étude du profil protéique des plantules de tomates se rapprochaient de ceux obtenus lors des analyses d'expression génique et est conforme aux résultats obtenus par d'autres auteurs (Osuna *et al.*, 2007; Price *et al.*, 2004; Thimm *et al.*, 2004). Plus précisément, l'étude du protéome des plantules *in vitro* nous a permis d'identifier plusieurs protéines impliquées dans la croissance cellulaire, le stress oxydatif et la photosynthèse. Nous avons observé une augmentation de l'abondance de protéines impliquées dans l'élongation, le repliement et la dégradation des protéines dans le cytoplasme, la mitochondrie, et le chloroplaste en présence de saccharose. Des données photosynthétiques ont permis d'observer une diminution du point de saturation lumineux de

la photosynthèse et de l'efficacité quantique et une augmentation du point de compensation lumineux chez les plantules en présence de saccharose exogène. Dans cet ordre d'idée, il semble que le saccharose limiterait la photosynthèse en partie par l'altération du flux de la chaîne de transport d'électron, par la limitation de la disponibilité du CO_2 et la modification du contenu en Rubisco activase. De plus, nous avons observé une modification de l'abondance de deux enzymes impliquées dans le métabolisme des espèces réactives à l'oxygène. Plus particulièrement, nous avons constaté une diminution et une augmentation de l'accumulation de la Cu/Zn superoxide dismutase (Cu/ZnSOD) et de la glutathione peroxidase (GPX), respectivement. La diminution de l'abondance de Cu/ZnSOD est possiblement attribuable à la faible photosynthèse (Dugas & Bartel, 2008; Slesak *et al.*, 2006) alors que l'augmentation de l'abondance de GPX est vraisemblablement imputable à l'augmentation de la capacité antioxydante (Couée et al., 2006) et/ou l'excès d'électrons générés par la chaîne de transport d'électron mitochondriale des plantules cultivées en présence de saccharose exogène. Nos données suggèrent que les plantules de tomate, en présence de saccharose exogène, présentent des caractéristiques similaires à celles de feuilles de peuplier lors à la transition de l'hétérotrophie à l'autotrophie au cours de leur développement. En particulier, la photosynthèse et la synthèse de parois secondaires sont inhibées et les protéines ribosomales ainsi que la synthèse protéique sont stimulées tel qu'observée chez les jeunes feuilles de *Populus tremula* (Sjodin *et al.*, 2008).

Nos résultats montrent que la présence de saccharose exogène dans le milieu MS (qui contient une forte teneur en N) stimule la synthèse protéique des plantules tel que pressentis dans notre hypothèse initiale. Nous avons rapporté l'augmentation et la diminution de l'accumulation de 58 et 7 protéines, respectivement, lorsque du saccharose était ajouté au milieu de culture. De plus, parmi les protéines affichant une plus grande abondance en présence de saccharose, plusieurs étaient impliquées dans la synthèse protéique en général. À l'opposé, il ne semble pas que l'apport de sucre stimule l'assimilation de l'azote, puisqu'aucune protéine impliquée dans ce processus n'a été identifiée parmi les 17 protéines analysées par spectrométrie de masse.

Il semble que le sucre exogène procure des effets bénéfiques sur la croissance des plantules, particulièrement en ce qui a trait à la division cellulaire et possiblement

l'accroissement de la surface foliaire (Cookson *et al.*, 2005; Granier *et al.*, 2000). Néanmoins, les sucres exogènes semblent laisser une empreinte négative sur la capacité photosynthétique et perturbent le métabolisme des ROS. On devrait par conséquent mieux étudier l'impact des changements provoqués par le saccharose, plus particulièrement au niveau de la division cellulaire et du métabolisme des ROS, sur l'acclimatation des plantules. Afin de bénéficier des avantages du saccharose et d'éviter ces désagréments, on devrait envisager de mettre au point un système de culture faisant place à une acclimatation *in vitro*. Cela inclurait probablement une diminution de la concentration de sucre, avant de procéder à l'acclimatation *ex vitro*. Pour implanter cette nouvelle étape, les plantules devront nécessairement être mises en culture sur milieu liquide afin de faciliter le changement des conditions avec saccharose aux conditions sans saccharose. Inévitablement, l'étape d'acclimatation *in vitro* devrait aussi inclure un meilleur renouvellement en CO_2 dans le contenant de culture et une augmentation de la luminosité afin d'augmenter la capacité et l'activité photosynthétique des plantules et limiter les dommages oxydatifs qui pourraient survenir dans le chloroplaste lors du transfert *ex vitro*. Le principe de l'acclimatation *in vitro* n'est pas nouveau. Des études ont évalué l'impact *in vitro* de la réduction de l'humidité relative, de l'utilisation d'antitranspirant et d'agent inducteur du système de défense sur la survie à l'acclimatation (Nowak & Shulaev, 2003; Pospisilova, 1996). L'efficacité d'un tel protocole, basé sur l'adaptation séquentielle des plantules *in vitro*, se doit d'être investiguée.

Antérieurement, l'information sur les processus physiologiques et biochimiques obtenue par les techniques traditionnelles a permis de mieux comprendre la croissance et le développement des plantules en conditions *in vitro*. Cependant, la culture *in vitro* est un système complexe et artificiel qui gagne à être étudié d'un point vue holistique. Pour aborder la problématique de l'acclimatation des plantules *in vitro*, nous avons décidé d'analyser globalement la réponse des plantules aux conditions de culture *in vitro*, et ce, à différents niveaux métaboliques. Cette approche possède l'avantage de générer rapidement des données sur des centaines de gènes afin d'obtenir un portrait métabolique complet. Cependant, nous devons être prudents dans l'interprétation des résultats d'expression génique puisqu'ils ne sont pas toujours corrélés avec le contenu en protéines, l'unité fonctionnelle des gènes. Par exemple, dans le cas où nous observons une expression plus

faible dans un traitement, nous ne pouvons savoir si l'expression est diminuée due à un besoin plus faible en cette protéine ou à l'inverse, que l'accumulation de la protéine cause une rétroaction négative sur son expression. Il importe de juxtaposer des résultats de transcriptomique et de protéomique afin d'obtenir une image plus juste des processus physiologiques qui se produisent. Ainsi, il est difficile d'émettre des conclusions définitives et des études supplémentaires seront nécessaires afin de confirmer les implications métaboliques et pratiques des résultats obtenus dans le cadre de cette thèse. Dans la conception même du projet, nous étions conscients des avantages et limites de notre approche, mais notre but principal était de contribuer à l'étude du phénomène par de nouveaux résultats nous permettant d'émettre de nouvelles hypothèses sur les mécanismes impliqués dans les problèmes d'acclimatation des plantules.

En résumé, les données obtenues dans le cadre de ce projet permettent de mieux définir l'impact des conditions de culture *in vitro* et du saccharose exogène sur le métabolisme des plantules en culture. Nous avons observé un effet marqué des conditions de culture *in vitro* sur le système de défense des plantes, le métabolisme des espèces réactives de l'oxygène (statut oxydatif de la plante), la force des puits, l'assimilation de l'azote, la synthèse protéique, la photosynthèse et la division cellulaire. Par ailleurs, la carence en sucre imposée aux plantules entraîne, non pas une réponse de famine, mais plutôt une transduction agissant sur la voie signalétique de l'éthylène et menant à une altération de la paroi cellulaire. Globalement, la perturbation du métabolisme des espèces réactives de l'oxygène, de la paroi cellulaire et du développement et de la division des cellules sont des réponses prédominantes chez les plantules en culture *in vitro*. Ces réactions sont cohérentes avec le phénotype observé en culture *in vitro* et leur impact sur l'acclimatation devra nécessairement être mesuré si l'on veut améliorer l'efficacité du transfert *ex vitro*.

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Annexe 1 : List of metabolically-classified gene present on the custom cDNA microarray

Annotation and functional classification	Genbank accession
A. Defense-related	
1. Cysteine proteinase inhibitor 7 precursor	BF050929
2. Pathogenesis-related protein (ATOZI1)	AI490707
3. Pathogenesis related protein (P4)	M69247
4. Pathogenesis-related leaf protein 6 precursor (P6)	AW035574
5. Pathogenesis-related protein (STH)	AW029723
6. Putative pathogenesis-related protein (PVPR3)	AW032481
7. Pathogenesis-related protein osmotin precursor (NP24)	AW035171
8. Pathogenesis-related protein (LRP)	AW036200
9. Pathogenesis-related protein (PR-1a1)	AW218809
10. Pathogenesis-related protein (PR-5)	AJ277064
11. Pathogenesis-related protein (PR-P23)	AW218786
12. Pathogenesis-related protein (PR1a2)	Y08844
13. Protein inhibitor II	X94946
14. CathDInh gene for Cathepsin D Inhibitor	AJ295638
15. Cathepsin D inhibitor protein	X73986
16. Serine protease (SBT1)	X98929
17. 14-3-3 protein (TFT7)	X95905
18. 14-3-3 protein (TFT6)	X95904
19. 14-3-3 protein (TFT5)	X95903
20. 14-3-3 protein (TFT3)	X95902
21. 14-3-3 protein (TFT2)	X95901
22. Leucine aminopeptidase (LAP)	U50151
23. Ascorbate peroxidase TL29	AJ251882
24. Glutathione reductase, chloroplast precursor	AW034391
25. Allene oxide synthase (AOS)	AF230371
26. Allene oxide synthase (AOS)	AJ271093
27. Cytochrome P450 CYP74C3	AF454634
28. Lipoxygenase (LOX)	U13681
29. Lipoxygenase	X94945
30. Lipoxygenase (LOXC)	U37839
B. Signal transduction and transcription	
1. MAP kinase phosphatase (MKP1)	AF312747
2. Putative protein kinase (LESK1)	AF230198
3. LSTK-1-like kinase	AF079103
4. Calcium dependent protein kinase (CPK1)	AJ308296
5. Serine/threonine protein kinase pk23	AY079049
6. Protein tyrosine phosphatase	AJ313509
7. DNA-binding protein (PTI5)	U89256
8. DNA-binding protein (PTI6)	U89257
9. DNA-binding protein (PTI4)	U89255
10. SNF1-related protein kinase (SnRKs)	AF143743
C. Carbon metabolism	
I. Sucrose metabolism	
1. Putative sucrose synthase	BI203222
2. Sucrose-phosphate synthase	AF071786
3. Sucrose transporter (SUT4)	AF176950
4. Beta-fructosidase (LIN5)	AJ272304
5. Invertase	E16293
6. Acid invertase	AB004558
7. ADP-Glucose pyrophosphorylase (AGPase)	U88089

8. Trehalose-6-phosphate synthase (TPS)	AW034662
9. Trehalose-6-phosphate phosphatase (TPP)	AW031718
10. Triose phosphate translocator	X92656
II. Cellular respiration	
1. Pyruvate kinase (PK)	AW035242
2. Hexokinase (AtHXK1)	AJ401153
3. Fumarase	AW034595
4. Succinate dehydrogenase (SDH3)	AF362730
5. NAD-dependent isocitrate dehydrogenase (ICDH)	Y16126
6. Glucose-6-phosphate dehydrogenase (G6P)	AW035603
7. ATP synthase beta subunit, mitochondrial precursor	AW031780
8. Phosphoenolpyruvate carboxylase (PEPcase)	AJ243416
9. Phosphoenolpyruvate carboxylase kinase (PCK)	AY190084
10. Malate dehydrogenase (MDH)	L27509
11. Fructose biphosphatase (FBP)	AJ004921
12. Glycine hydromethyltransferase	AW033233
D. Energy metabolism (photosynthesis)	
1. ATP synthase beta subunit (ATPB)	AJ236183
2. Photosystem II oxygen-evolving complex	AI484134
3. Photosystem II 23 kDa protein	BG123457
4. Photosystem II stability/assembly factor HCF136	BG123171
5. Photosystem II reaction center 6.1 kDa protein	AW038772
6. Photosystem II 10 kDa polypeptide precursor	AW037270
7. Photosystem i reaction centre subunit x precursor light-harvesting complex i 7 kDa protein) (PSI-K)	BF052204
8. Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RBCL)	AF479571
9. Rubisco activase	AI484371
10. Chloroplast NADH dehydrogenase subunit (NDHF)	U08921
E. Nitrogen metabolism	
1. Nitrate reductase (NR)	BG791271
2. Nitrite reductase (NiR)	BG791272
3. Glutamine synthetase (GS)	U15059
4. Glutamate synthetase (GOGAT)	AW032148
5. Nitrate transporter 1 (NRT1.1)	X92853
6. Asparagine synthetase (AS)	AY240926
F. Growth and development	
1. β-galactosidase (TBG4)	AF020390
2. Beta-galactosidase (TBG7)	AF154422
3. Expansin 18 (EXP18)	AJ270960
4. Xyloglucan endotransglycosylase (BR1)	AF205069
5. Endo-1,4-β-glucanase precursor (Ce12)	U13055
6. Endo-1,4-β-glucanase precursor (Ce11)	U13054
G. Protein folding and degradation	
1. 26S proteasome regulatory subunit S2	BG130976
2. 26S proteasome regulatory subunit S3	BG123451
3. 20S proteasome beta2 subunit	BF051920
4. Proteasome alpha subunit (5' prime sequence)	AW224522
5. 20S proteasome beta subunit (PBG1)	AW218173
6. 20S proteasome beta subunit (PBG1)	AW217562
7. 26S proteasome subunit 7	BE441156
8. Proteosome α-subunit	BG589315

9. Tat binding protein homolog	BF096286
10. Cysteine protease TDI-65 (TDI-65)	AF172856
11. Serine protease (SBT4A)	AJ006377
12. 20S proteasome β-subunit (PBC2)	BG791266
13. Cysteine protease (CYP1)	AW218147
14. Serine protease (SBT3)	AJ006376
15. Cathepsin B-like cysteine protease	AW154851
16. Subtilisin-like protease (P69C)	AW036296
17. ClpC protease	BF098481
18. ClpC protease 2 proteolytic subunit (CLPP2)	AJ308540
19. ATP-dependent clp protease	BF097665
20. Serine protease (SBT2)	X98930
21. ATP-dependent clp protease CD4B precursor	AW035654
22. Putative protease (SOHB)	AW034551
23. Ubiquitin-specific protease	AI777008
24. Auxin-induced proteinase inhibitor (ARPI)	BG130975
25. Ubiquitin (UBI3)	X58253
26. Endopeptidase complex β-subunit (5' sequence)	AW037263
27. Endopeptidase complex β-subunit (3' sequence)	AW037262
28. DnaJ like protein (T19-8)	AJ295232
H. Cellular function	
1. Myosin-like protein my5	BF050314
2. Rab11 GTPase	AJ245570
3. Tomato actin (TOM51)	BF096262
4. Actin	BF098335
5. Actin depolymerizing factor	BG791215
6. Profilin	AJ417553
I. Others	
1. Tomato demethylated	BH012224
2. Glucosyltransferase, immediate-early salicylate-induced,putative	AW029693
3. Acid phosphatase	AF305968
4. Ran binding protein-1	CD002747