禽细胞因子的新功能——免疫治疗和疫苗佐剂

孙建和* 陆 萃
(上海交通大学农业与生物学院,上海 201101)

摘 要 许多国家已明令禁止在饲料中使用抗生素类饲料添加剂和化学抗菌药物,从而使得肉品生产者急于寻求新的替代产品。细胞因子是一种在感染或免疫接种后产生的蛋白质,能够影响免疫应答的类型和水平,因此是一种绝佳的天然替代产品。随着更多的禽细胞因子基因的发现,临床应用细胞因子成为可能。由于禽类的免疫系统与哺乳动物的相似,因此相关工作也为研究细胞因子在控制家畜疾病上提供了颇具前景的动物模型。这里综述了禽细胞因子的最新研究进展,并侧重阐明了细胞因子作为治疗制剂和疫苗佐剂的功能与前景。

关键词 禽细胞因子,免疫治疗,疫苗佐剂
中图分类号 Q952.6 文献标识码 C 文章编号 1000-3061(2003)02-0141-06

应用抗生素控制畜禽疾病已有 50 多年的历史,抗生素还是生长促进剂,在饲料中添加微量的抗生素能提高动物生长率和饲料报酬。多年来,医学上抗生素抗性细菌的出现就怀疑与家畜饲料中的抗生素有关。在家禽上,长期应用化学药物控制细菌性疾病和寄生虫病也产生了一系列问题。例如,艾美球虫属的某些虫株就对化学药物产生了抗性,因此很难治疗。此外,肉品中的化学物质残留、通过水的径流造成化学物质污染环境等也是个重大问题。

1986 年,瑞典率先禁止在食用动物上使用抗生素类生长促进剂[1]。欧盟也禁止在饲料添加剂中使用 4 种抗生素作为生长促进剂,1998 年进一步禁用预防性抗生素。世界卫生组织也强烈要求应用不对环境造成危害的产品替代抗生素来控制疾病。其他国家随后也都颁布了相应的法规来控制抗生素在食用动物上的应用。

随着日益广泛的饲料中抗生素和化学药物的禁用,为了不给畜禽产业带来新的负面影响,必须寻找替代产品,因为如果没有预防性抗生素的替代物,那么暂时被抑制的微生物就可能复活并致病。基于以往的经验,不难预测在养禽业上本来可用抗生素控制的产气荚膜梭菌的感染将会增加,从而导致胃肠道问题增多、肉品质量下降。因此,发掘家畜生长促进剂的替代品非常迫切。

1 抗生素类生长促进剂的替代物

抗生素和化学药物的替代物必须安全、使用方便、价廉、高效。尽管已有许多替代方法,但大多数都不尽如人意。一种方法是在饲料中添加酶[2],如 Avizyme,它能提高日粮的消化率和糖的供给,最终改善饲料的质量以及增加能被肠道菌群利用的底物数量。另外一种方法是使用甜菜碱,它能减轻肠道寄生虫导致的肠道损伤,并可抑制产气荚膜梭菌的二次感染,从而降低坏死性肠炎的发生。此外,由某些细菌产生的小分子蛋白质——细菌素[3],能清除其他竞争菌群。特别值得一提的是,已发现某些类型的细菌素能特异性杀死导致鸡坏死性肠炎的细菌。应用基因工程技术获得重组细菌素将为替代抗生素、控制产气荚膜梭菌以及其他鸡致病菌繁殖提供较好的替代产品。

鸡细胞因子则是另一种最有应用前景的家禽饲料抗生素的替代物,细胞因子是在感染或免疫接种后由免疫系统产生的蛋白质。细胞因子可通过介导多种效应包括免疫细胞的激活或分化、诱导其他细胞因子的产生来调节免疫应答,从而增强免疫功能。

2 禽细胞因子基因的克隆

鸡的细胞因子基因的发现、克隆与定性在一定程度上滞后于哺乳动物相关的研究工作。由于序列同源性普遍较低(约 30% ~ 50%),因此,在分离哺乳动物细胞因子鸡的同源物方面的进展较慢。应用同源探针进行识别和应用基于哺乳动物序列的引物进行 PCR 扩增对应的禽细胞因子基因通常很难成功,而与哺乳动物基因具有较高同源性的基因可以用该方法进行克隆,如干细胞因子。尽管总的说来同源性很低,但应用哺乳动物中同源性高度保守的一小段设计引物,

收稿日期:2002-08-27,修回日期:2002-11-18。
基金项目:上海市科学技术发展基金资助(No.01JC14034)。
* 通讯作者。 Tel:86-21-64789734;Fax:86-21-64193344;E-mail:sunjianhe999@163.com

也成功克隆了 α-干扰素基因。其他方法如基于功能表达从 cDNA 文库中识别克隆的技术也已成功建立。应用该方法已经发现了 3 种鸡细胞因子基因:鸡 γ-干扰素基因[4]、鸡白介素-1β[5] 和鸡白介素-2[6]。

最近,禽细胞因子基因克隆方面的进展在于表达序列标签(Expressed sequence tag,EST)文库的建立。ESTs 是短的单通道的 DNA 序列,来源于 cDNA 克隆的任一端,尽管这些 ESTs 相对较短,一般不超过 500bp,但这一大小的序列通常足以获得大多数细胞因子基因的开放读框(ORF),许多研究小组已经测定了来源于不同类型细胞的 ESTs 的鸡 cDNA 序列。禽类免疫学家已建立了包含这些 EST 序列的基因库,若需获得更多相关信息,请查看如下网站:http://www.chickest.udel.edu;http://genetics.hpi.uni.-ham-burg.de/estonline.html。

在进化相对独立的生物中,通过单一的序列比较通常很难发现细胞因子同源物。在这种情况下,基因组学已成功应用于禽细胞因子基因的识别。通过像测定启动子保守区上游序列一样测定外显子与内含子的比例及核苷酸在每个区域的数量,将可能根据其与哺乳动物的同源性来发现禽细胞因子基因。该方法已成功应用于从鸡白介素-15 的同源物中发现了鸡白介素-2,并帮助确定了鸡白介素-8 基因的存在[7]。也有学者应用该技术对不同禽品种的 γ-干扰素的外显子结构进行比较,揭示了这些基因的进化关系。检测基因结构、构建细胞因子基因在特定染色体上的图谱,将对定性新的禽细胞因子基因具有关键作用。

3 禽细胞因子

根据细胞因子产生应答的类型,通常可分为 Th1 型和 Th2 型细胞因子。Th1 型细胞因子包括 IL-2、IFN-γ、肿瘤坏死因子(TNF)和淋巴毒素(LT),其主要与细胞免疫应答有关。IFN-γ 和 TNF 是巨噬细胞活化细胞因子,而 LT 则直接对某些细胞产生细胞毒性。Th2 型细胞因子主要与 B 细胞活化有关,因此,主要调节抗体的产生。IL-4、IL-5、IL-6、IL-10 和 IL-13 属于 Th2 型细胞因子,其功能主要是调节体液免疫应答。迄今已克隆的鸡的细胞因子基本上都属于 Th1 样,只有最近发现的 IL-6 除外[8]。其他经典的 Th2 型细胞因子是否在鸡中也存在还有待进一步验证。

目前至少已经克隆了 16 种鸡细胞因子基因,并且其中的一些在其他品种的禽类中也已克隆成功,包括 火鸡、日本鹌鹑、鸭、雉鸡和珍珠鸡等。鸡与其他品种家禽的氨基酸同源率高,因此应用对应的鸡的序列来设计引物,通过 PCR 克隆这些细胞因子基因就相对容易。一旦克隆,就可定性其基因产物并评估其免疫增强活性。从事细胞因子研究的许多实验室最近组成了禽细胞因子研究组(Avian Cytokine Group,ACG),以便于信息和成果的交流。一些实验室已被指定作为某些特定细胞因子的参照实验室,将为实验对照提供国际标准。网址如下:http://www.geel.li.csiro.au/aviancytokines.

3.1 鸡 γ-干扰素(IFN-γ)

鸡 γ-干扰素是研究得较广泛的鸡细胞因子之一。由于鸡 γ-干扰素与哺乳动物的 γ-干扰素具有共同的特性,使得发现这一基因相对容易[4]。IFN-γ 是细胞因子干扰素家族的一个成员,其具有调节免疫应答和抑制病毒繁殖的能力,有了这些特性,IFN-γ 作为免疫增强剂和疫苗佐剂的能力就显而易见了。

在养禽业,由于超强毒株的出现,使得活疫苗的应用受到很大关注,要求寻找替代疫苗。然而,灭活疫苗或重组亚单位疫苗不能提供足够的保护,通常需要使用佐剂。但是油佐剂通常会诱导局部负反应,导致肉品质降低,因此在家禽上没有合适的价廉、高效的佐剂。当与抗原一起免疫鸡体时,鸡 γ-干扰素是一种具有增强抗体应答能力的细胞因子。与单独注射抗原相比,重组鸡 γ-干扰素能诱导产生持久的高水平的二次抗体应答[9]。

同样,应用鸡球虫病攻击模型,鸡 γ-干扰素的免疫增强和治疗作用也已得到广泛证明[10]。应用鸡 γ-干扰素治疗感染了艾美尔球虫的鸡能获得保护并且能降低该病导致的体重减轻。鸡 γ-干扰素还是一种天然的生长促进剂,使用后能提高体重 3% ~ 8%。

3.2 ChIL-1β

IL-1β 在哺乳动物会诱导一系列负反应,例如发热、皮质酮升高以及细胞因子网络普遍激活。哺乳动物 IL-1β 能经 IL-2 诱导,刺激 T 细胞增殖以及诱导 B 细胞成熟和抗体产生。用鸡巨噬细胞系的 cDNA 表达文库完成了鸡 IL-1β 基因的鉴定和克隆[5],重组的鸡 IL-1β 呈现与哺乳动物同源物相似的生物学活性,即能诱导成纤维细胞分泌化学因子、上调皮质酮的产量。应用破伤风类毒素作为抗原,证明鸡 IL-1β 具有免疫佐剂活性[11],与应用单独的抗原接种相比,当用重组蛋白接种时,鸡 IL-1β 能提高抗体应答水平,共接种鸡的 IL-1β、IFN-α 和 IFN-γ,其对破伤风类毒素的抗体应答呈现累加效应。这表明将细胞因子合用作为佐剂可能更有效。

3.3 ChIFN-α

鸡 α-干扰素最初克隆于鸡的成纤维细胞[12],I 型和 II 型 IFN(IFN-α/β)都具有抗病毒和抗肿瘤效应,并作为免疫调节剂发挥重要作用。应用重组鸡 α-干扰素通过饮水免疫 1 日龄雏鸡,并在免疫后 1d 用新城疫病毒进行攻击,以估测其疗效和抗病毒活性。与对照相比,免疫了高剂量重组鸡 α-干扰素的鸡群,平均体重更高、昏睡的鸡更少,更为重要的是,免疫过的鸡群能完全防止病毒在气管中繁殖。进一步研究发现将 NDV 和 ChIFN-α 在禽痘病毒载体(FPV)中共表达,鸡群对 NDV 的攻击能提供保护。用 NDV 基因由 FPV 介导注射鸡体,同样能诱导产生很好的保护作用,但是由于 FPV 的缘故,鸡的增重会有影响。当用 ChIFN-α 与 FPV 中的 NDV 基因共注射时,与对照相比,体重不会进一步降低,表明 ChIFN-α 在保持免疫效果的前提下能用于控制某些禽类疾病。ChIFN-α 与 ChIFN-γ 联合应用的抗病毒效果有待进一步研究。

3.4 ChIL-15 和 ChIL-18

ChIL-15 和 ChIL-18 的基因只是在最近才有报道[13]。在

哺乳动物,由于 IL-15 与 IL-2 的受体组成相似,所以二者在体外具有许多相似的功能,包括 T 细胞增殖。然而,由于这两种细胞因子以独特的 α 链结合到受体上,使得二者在体内能介导完全不同的功能。IL-15 在哺乳动物一个主要的功能为调节 NK 细胞的发育与增殖,通过 NK 细胞产生 IL-15 共刺激细胞因子从而调节巨噬细胞和 NK 细胞间的相互作用。鸡 IL-15 在活化 NK 细胞和 $CD8^+$ 记忆 T 细胞上也发挥了主要作用,因此也具备作为疫苗佐剂的潜能。

在哺乳动物 IL-18 也是由活化的巨噬细胞产生的[14]。其基本功能是通过 Th1、NK 细胞和 NK T 细胞上调 IFN-γ 的产量。IL-18 也能作用于 T、B 和 NK 细胞,诱导各种其他细胞因子的产生。

3.5　ChIL-2

多年前,在鸡中就发现具有 IL-2 样特性的 T 细胞生长因子,然而几年前才对其基因进行了克隆[6]。由于在鸡和哺乳动物间存在一定的进化距离,应用哺乳动物的 IL-2 探针杂交筛选鸡 cDNA 文库的工作未能成功。从用 ConA 激活的脾细胞上清中也未能获得足以测序的高纯度的 ChIL-2。最后,从一个活化的鸡脾 cDNA 文库中克隆到一种鸡的淋巴因子基因,其表达产物呈现 T 细胞增殖活性,该基因与哺乳动物的 IL-2 和 IL-15 都具有同源性,其中与牛 IL-2 和 IL-15 的氨基酸的同源性分别为 24% 和 25%。这一发现非同寻常,因为曾有报道在哺乳动物的 IL-2 和 IL-15 间,无论在核苷酸水平还是在氨基酸水平都没有显著的同源性。这种鸡细胞因子与哺乳动物的 IL-2 也具有相似的特性,通过活化的 T 细胞专一性表达含有 20 个核苷酸的 5′端非翻译区并含有一个短的信号肽。然而,IL-15 存在 4 个特征性的保守的半胱氨酸,这在 IL-2 是不存在的。基因结构分析表明该基因是哺乳动物 IL-2 的鸡的类似物,与 IL-15 相比,基因结构更象 IL-2,具有 4 个外显子和 3 个内含子,与人和小鼠的 IL-2 基因相似[7]。启动子也与哺乳动物 IL-2 非常相似,大部分的转录因子结合位点无论在核苷酸序列还是在排列上都很保守。该基因位于鸡的 4 号染色体上。氨基酸序列比较发现 ChIL-2 与哺乳动物的对应部分具有相似的蛋白结构。

哺乳动物的 IL-2 是免疫应答所必需的细胞因子,包括 T 细胞的分化和激活、B 细胞的发育以及 NK 细胞的激活等。据此,有学者研究了 ChIL-2 在体内和体外对免疫细胞群的影响。ChIL-2 在体外呈现与哺乳动物 IL-2 相似的生物学活性,在 ConA 刺激后,能诱导 T 细胞增殖,这一结论通过加入针对 ChIL-2 受体 α 链的单克隆抗体封闭其活性得到了进一步证实[15]。而且,还发现了 ChIL-2 发挥活性的靶细胞。新分离的 $CD4^+$ 和 $CD8^+$ 脾细胞表达 ChIL-2R 的比例较低,但激活后,在 24h 内,绝大部分的 $CD4^+$ 细胞能表达 ChIL-2R,但 $CD8^+$ 细胞表达受体却延迟了。

由于哺乳动物的 IL-2 是有效的疫苗佐剂,那么鸡的 IL-2 是否也有同样的作用呢?有学者对 ChIL-2 接种后在体内的残留时间进行了研究,静脉注射后 ChIL-2 的血清浓度在 1 ~ 2min 之内达到峰值,随后迅速下降,半衰期小于 3min。这种快速清除的特性是许多细胞因子的共同特征,表明重组 ChIL-2 既能快速降解也能迅速与靶细胞的 ChIL-2R 结合。尽管 ChIL-2 被快速清除,但它能导致 48h 内 $CD4^+$ 和 $CD8^+$ 外周血 T 细胞比例升高[16]。总之,从 ChIL-2 所呈现的生物学活性上来看,当其与疫苗一起接种时,可能会增强细胞免疫应答。

3.6　ChIL-6

IL-6 在哺乳动物具有广泛的活性,几乎能影响免疫系统的所有细胞。其主要活性是影响急性期的蛋白应答,但也会干扰 Th2 型应答的产生。最近刚克隆了 ChIL-6 基因[8],并对其生物学特性进行了阐述,这一成果可谓是禽细胞因子研究领域的一个重大突破,因为在这之前克隆的禽细胞因子主要影响 Th1 型应答。该成果为研究 ChIL-6 对 IgA 抗体应答产生的影响提供了可能。IL-4、IL-5 和 IL-10 等细胞因子在哺乳动物 Th2 型应答的产生上十分重要,但在禽类尚未发现其类似物。

4　禽细胞因子的接种

全世界每年生产肉鸡 400 亿只,细胞因子如果要应用于商业化治疗,那么免疫接种和治疗的方法则应是首要考虑的因素,其必须安全、方便、经济、高效。用杆状病毒、酵母和大肠杆菌等表达系统生产的重组细胞因子通过注射已成功免疫了大型经济动物如猪和牛等,若要在鸡上应用,重组蛋白则必须进行规模生产,以降低成本。应用表达细胞因子和疫苗的活病毒载体进行接种已取得成功并且无须多次加强免疫接种。活病毒载体如表达细胞因子基因的禽腺病毒(FAV)能克服重组细胞因子在体内半衰期短的缺陷,因为细胞因子在许多天内都能持续表达直到病毒被清除[17]。FAV 可经饮水或气雾进行接种,使接种过程大为简化。FAV 载体还有一些优点,其表达的蛋白比原核系统表达的蛋白更接近天然蛋白,因而活性更高。此外该病毒为鸡特异性病毒,不会在其它品种的动物中繁殖,因而更安全。最近,有应用 FAV 重组表达 ChIFN-γ 成功的报道。应用 FAV-ChIFN-γ 免疫商品鸡,鸡体重比对照组提高,即使是在感染了鸡球虫病的情况下也是如此[15]。

5　展　望

国内目前已克隆了鸡 IFN-γ[18]、鸭 IFN-γ[19]、ChIL-2[20] 和 ChIL-15 等细胞因子,属于 Th1 型细胞因子,对 ChIL-6 的研究尚未见涉猎,本实验室根据 GenBank 中发表的唯一 ChIL-6 基因序列,设计引物,分别用鸡传染性法氏囊病病毒感染和用 ConA 免疫刺激健康鸡,无菌取处理鸡的脾脏,匀浆后用 Trizol 抽提细胞总 RNA,应用随机引物反转录获得 cDNA,经 PCR 扩增获得安卡与岭南黄杂交后代的 ChIL-6 基因,应用 pGEM-T 载体克隆该基因,经测序,表明其与 GenBank 中公布的 ChIL-6 基因为同源基因。将该基因与鸡传染性法氏囊病病毒的主要保护性抗原基因串联后克隆入真核表达载体,经肌肉注射免疫鸡体后,产生了较好的免疫应答反应。将

ChIL-6 基因表达产物作为鸡生长促进剂和抗生素的替代产品的研究目前正在进行。

研究细胞因子作为抗炎症和免疫刺激因子将是一个新兴且很有前景的研究方向。众所周知,应激和疾病等通常会影响动物的生长,通过降低应激和控制疾病可将体内的资源更多地应用于增重,在免疫系统、神经系统和内分泌系统的相互作用中,细胞因子可能发挥着调节的枢纽作用。有学者已开展实验以评估某些禽类细胞因子的促生长活性,与对照相比,注射了细胞因子的雏鸡呈现显著的增重效应,增重幅度达 3% ~8%,因此,细胞因子作为一种有效、天然的生长促进剂将具有广阔的前景[21]。

研究发现,作为治疗制剂,ChIFN-γ 在体外能激活巨噬细胞从而阻止艾美尔球虫的发育,并且在体内也具有杀球虫的潜能。由于 ChIFN-γ 具有抗感染和增强疫苗免疫效果的能力,因此其为治疗制剂和疫苗佐剂的最佳侯选之一,其他细胞因子也可能是很好的治疗制剂,如 TGF-β 和 cMGF 具有增强粘膜免疫、I 型干扰素具有抗病毒等治疗活性[22],说明细胞因子作为治疗制剂也具有美好的未来。

尽管部分细胞因子已用于体内调节免疫应答,但在临床应用方面仍相对滞后,因而这一技术仍然存在着巨大的开发应用潜力。虽然细胞因子的表达和纯化比较昂贵,并且需要多次注射,但倘若将其与 DNA 疫苗综合考虑的话,由于注入的质粒 DNA 在体内相当长的时间内都会表达细胞因子,因此当细胞因子被应用于调节 DNA 疫苗的免疫应答时,就具有非常明显的优势。虽然蛋白疫苗已存在较好的佐剂,但对于 DNA 疫苗却少有有效的方法来调节其免疫应答。在过去的几年中,应用细胞因子、趋化因子以及共刺激分子作为 DNA 疫苗的分子佐剂的研究报告很多。而大量的研究侧重于将诱导 Th1 型应答的细胞因子与 DNA 疫苗结合起来研究,大多数的研究结果发现 IgG1/IgG2a 的比率随着同时注射 IFN-γ,IL-2,IL-12,IL-15 或 IL-18 编码质粒而下降,大多数的 DTH 应答和 CTL 应答也增强。DNA 疫苗与编码诱导 Th2 型应答的细胞因子的质粒一起注射,诱导的免疫应答通常以产生高水平的 IgG1 抗体为特征,导致高水平的总抗体和高 IgG1/IgG2a 抗体比率。

虽然对细胞因子如何调控免疫系统以及如何优化其对疫苗的免疫应答等都有了一定的认识,但仍存在较多问题。问题之一是对保护性免疫应答的本质尚不甚清楚。在哺乳动物,对病原的获得性保护通常是由于细胞免疫或体液免疫在起作用,然而,细胞免疫、体液免疫以及先天性免疫应答会在感染的不同阶段产生,要确定何种应答决定保护非常困难。在鸡方面,尚不清楚是否也存在象哺乳动物那样的 Th1/Th2 模式。为了合理设计对某种疾病的治疗方法,关键的一步是要弄清保护性免疫应答的本质。这就要求在病原感染过程中研究细胞因子的产生。随着发现的禽细胞因子的不断增多,以及实时 PCR 和 TaqMan 技术的建立,使得对感染过程中细胞因子产生曲线的精确绘制成为可能。

现存的另一问题是细胞因子如何接种家禽的技术问题。对于商业化的养鸡业,注射接种重组细胞因子蛋白显然不太合适,此外,由于细胞因子在体内会被迅速降解和清除,故需多次注射,这更是行不通的。因此有必要进一步优化免疫接种策略。研究发现细胞因子等分子佐剂会调节来源于不同病原的抗原的免疫应答,因此其应用前景非常广阔。在某些情况下,免疫途径会影响分子佐剂的效果,如对于 HSV 抗原,经鼻或眼注射 IL-4 能提高保护,而经肌肉注射,尽管对免疫指标的影响相似,但不能诱导保护。这一发现提示在评估 DNA 疫苗的效果时免疫指标的测定不能替代攻击试验。由于免疫接种途径的不同而导致细胞因子作用上的差异表明,这些细胞因子需要与存在于某一组织位点的细胞相互作用方能发挥作用。尽管这方面大多数的研究都用小鼠进行,但在其他一些品种如 非人灵长类和大型动物上的一些研究也获得相似的结果,预示着分子佐剂的基本原理适用于人和兽医的 DNA 疫苗。

研究活病毒载体与 DNA 联合免疫接种的策略将具有很好的前景,活病毒载体技术已应用于多种细胞因子的表达和给药,这为经饲料、饮水和喷雾给药提供了一条简单、高效、价廉的商业化的给药途径。同时,可考虑采用单细胞因子或多细胞因子与疫苗抗原共同给药。并且可选用某一病毒载体使得抗原和细胞因子能靶向到特异位点如肠道或呼吸道等,从而在正确部位能产生合适的免疫应答[23]。将几种细胞因子共接种以试图进一步调节免疫应答的研究已经开展,但遗憾的是,对联合应用几种分子佐剂后,对感染攻击的保护作用,至今尚未进行研究,但有研究显示,IL-12 和 GM-CSF 似乎是最有希望的,当共接种这两类细胞因子时,与单独提呈任一种细胞因子基因相比,免疫应答都得到加强。表明通过组合不同的细胞因子,提高 CTL 应答是可行的,但这种组合存在一定的极限,超过它,即使进一步增加分子佐剂,也不再能提高应答水平。

研究发现在大多数情况下,可将编码抗原的质粒与编码细胞因子的质粒根据需要组合使用,这一方法的优点在于抗原/佐剂可进行大量组合,而不需重新构建大量的抗原/细胞因子重组质粒。有实验显示应用同时编码抗原和细胞因子的质粒免疫接种,发现确保抗原和细胞因子在同一细胞表达似乎没有必要,然而 GM-CSF 和抗原编码的质粒尽管不必在同一时间进行注射但必须在同一位点进行注射。与应用单独的编码抗原和细胞因子的质粒相比,将 IFN-γ 或 IL-4 与卵清蛋白偶联后能提高这些细胞因子的功效。这些结果表明,虽然是否由同一细胞表达抗原和佐剂显得并不重要,但在免疫过程中,抗原和细胞因子共同免疫仍具有一定的优势。当然,由于在每一个新融合蛋白产生的过程中,细胞因子和/或抗原可能会出现不适当折叠,因此要特别注意其特征。

FAV(Fowl Adenovirus Vector,FAV)技术提供了一个简易、高效、廉价的商业化接种系统,细胞因子作为一种加强保护性免疫应答的天然佐剂,可以直接将蛋白或重组载体通过卵在孵化前接种到鸡胚,该过程可应用自动化的蛋注射系统来完成,使得工作量大大降低[23],因此,预测这项技术在未来

将具有很好的开发、应用前景。

鉴于鸡的免疫系统与哺乳动物的相似，且鸡容易饲养、价格低廉、繁殖快、易于大量处理，还拥有大量可以应用的研究成果如针对细胞表面标记的抗体、细胞因子基因和蛋白以及一些良好的疾病模型，进行大规模的相关试验成本低廉，因此鸡将为研究细胞因子在控制集约化养殖家畜的疾病上提供一个很有吸引力的模型。

REFERENCES(参考文献)

[1] Williams P E V. The European ban of the prophylactic use of antibiotics as growth promoters in animal nutrition: political and economic aspects. In: Proceedings of the Australian Poultry Science Symposium, 2001, pp.83 – 93

[2] Bedford M. Removal of antibiotic growth promoters from poultry diets: implications and strategies to minimise subsequent problems. *World's Poult Sci J*, 2000, **56**: 347 – 365

[3] Jack R W, Tagg J R, Ray B. Bacteriocins of gram-positive bacteria. *Microbiol Rev*, 1995, **59**: 171 – 200

[4] Digby M R, Lowenthal J W. Cloning and expression of the chicken interferon-gamma gene. *J Interferon Cytokine Res*, 1995, **15**: 939 – 945

[5] Weining K C, Sick C, Kaspers B *et al*. A chicken homolog of mammalian interleukin – 1 beta: cDNA cloning and purification of active recombinant protein. *Eur J Biochem*, 1998, **258**: 994 – 1000

[6] Sundick R S, Gill-Dixon C. A cloned chicken lymphokine homologous to both mammalian IL-2 and IL-15. *J Immunol*, 1997, **159**: 720 – 725

[7] Kaiser P, Mariani P. Promoter sequence, exon: intron structure, and synteny of genetic location show that a chicken cytokine with T-cell proliferative activity is IL2 and not IL15. *Immunogenetics*, 1999, **49**: 26 – 35

[8] Schneider K, Klass R, Kaspers B *et al*. Chicken interleukin-6. cDNA structure and biological properties. *Eur J Biochem*, 2001, **268**: 4200 – 4206

[9] Lowenthal J W, O'Neil T E, Broadway M *et al*. Coadministration of IFN-gamma enhances antibody responses in chickens. *J Interferon Cytokine Res*, 1998, **18**: 617 – 622

[10] Lowenthal J W, Lambrecht B, van den Berg T P *et al*. Avian cytokines-the natural approach to therapeutics. *Dev Comp Immunol*, 2000, **24**: 355 – 365

[11] Schijns V E, Weining K C, Nuijten P *et al*. Immunoadjuvant activities of *E. coli*- and plasmid-expressed recombinant chicken IFN-α/β, IFN-γ and IL-1β in 1-day and 3-week-old chickens. *Vaccine*, 2000, **18**: 2147 – 2154

[12] Sekellick M J, Ferrandino A F, Hopkins D A *et al*. Chicken interferon gene: cloning, expression, and analysis. *J Interferon Res*, 1994, **14**: 71 – 79

[13] Schneider K, Phehler F, Baeuerle D *et al*. cDNA cloning of biologically active chicken interleukin-18. *J Interferon Cytokine Res*, 2000, **20**: 879 – 883

[14] Sugawara I. Interleukin-18 (IL-18) and infectious diseases, with special emphasis on diseases induced by intracellular pathogens. *Microb Infect*, 2000, **2**: 1257 – 1263

[15] Stepaniak J A, Shuster J E, Hu W *et al*. Production and in vitro characterization of recombinant chicken interleukin-2. *J Interferon Cytokine Res*, 1999, **19**: 515 ~ 526

[16] Lowenthal J W, O'Neil T E, David A *et al*. Cytokine therapy: a natural alternative for disease control. *Vet Immunol Immunopathol*, 1999, **72**: 183 – 188

[17] Johnson M A, Pooley C, Lowenthal J W. Delivery of avian cytokines by adenovirus vectors. *Dev Comp Immunol*, 2000, **24**: 343 – 354

[18] LIU S W(刘胜旺), CHEN H Y(陈洪岩), KONG X G(孔宪刚) *et al*. Cloning and sequencing of Chicken Interferon-γ. *Chinese Journal of Veterinary Science*(中国兽医学报), 2000, **20**(3): 228 – 230

[19] YIU J E(尤健儿), HUANG L N(黄莉娜), WANG W Y(王文逸) *et al*. Cloning and expression of Chinese Duck Interferon-γ gene. *Acta Biochimica et Biophysica Sinica*(生物化学与生物物理学报), 2001, **33**(6): 707 – 712

[20] LI J R(李建荣), HUANG Y W(黄耀伟), MENG S S(孟松树) *et al*. Cloning and phylogenetic analysis of Interleukin-2 gene in Xiaoshan Chicken, a Chinese local chicken breed. *Acta Biochimica et Biophysica Sinica*(生物化学与生物物理学报), 2001, **33**(6): 713 – 718

[21] Lowenthal J W, York J J, O'Neil T E, Rhodes S, Prowse S J, Strom A D G, Digby M R. *In vivo* effects of chicken interferon gamma during infection with *Eimeria*. *J Interferon Cytokine Res*, 1997, **17**: 551 – 558

[22] Marcus P I, van der Heide L, Sekellick M J. Interferon action on avian viruses. I. Oral administration of chicken interferon-alpha ameliorates Newcastle disease. *J Interferon Cytokine Res*, 1999, **19**: 881 – 885

[23] Louise S H, Andrew G D B, John W L. The emerging role of avian cytokines as immunotherapeutics and vaccine adjuvants. *Veterinary Immunology and Immunopathology*, 2002, **85**: 119 – 128

The Potential of Avian Cytokines as Immunotherapeutics and Vaccine Adjuvants

SUN Jian-He* LU Ping

(*School of Agriculture and Biology, Shanghai JiaoTong University, Shanghai* 201101, *China*)

Abstract With the imminent and widespread ban of the use of antibiotic feed additives and chemical antimicrobials in food production animals, alternative measures need to be sought to ensure that the livestock industry will not be adversely affected. Cytokines are proteins that control the type and extent of an immune response following infection or vaccination. They therefore represent excellent naturally occurring therapeutics. The identification, cloning and characterisation of cytokine genes in chickens have lagged somewhat behind similar work in mammals. Progress in isolating chicken homologues of mammalian cytokines has also been slowed by the generally low level of sequence similarity. Chicken cytokine genes that have been cloned to date include ChIFN-γ, ChIL-1β, ChIFN-α, ChIL-15, ChIL-18, ChIL-8, ChIL-2, ChIL-6, ChIL-16, SCF, MGF, TGFβ, Lymphotactin, MIP-1β, CXC and CC chemokines, so the use of cytokines in poultry has become more feasible with the discovery of a number of avian cytokine genes. The delivery methods for chicken cytokine are of prime importance and are required to be safe, easy to administer and cost-effective. Live viral vectors such as fowl adenovirus (FAV) expressing cytokine genes can be delivered via drinking water or aerosol sprays, making it very easy to administer. Since the immune system of chickens is similar to that of mammals, they offer an attractive model system to study the effectiveness of cytokine therapy in the control of disease in livestock. This review focus on the recent advances made in avian cytokines, with a particular focus on their assessment as therapeutic agents and vaccine adjuvants.

Key words avian cytokines, immunotherapeutics, molecular adjuvants

Received: 08-27-2002

This work was supported by Grant from Shanghai Municipal Science and Technology Committee (No.01JC14034).

* Corresponding author. Tel: 86-21-64789734; Fax: 86-21-64193344; E-mail: sunjianhe999@163.com

《生物工程学报》第二届编委会名单

Identification and Expression Analysis of a Full-length cDNA Encoding a *Kandelia candel* Tonoplast Intrinsic Protein

HUANG Wei[1,2] FANG Xiao-Dong[2,3] LIN Qi-Feng[2*] LI Guan-Yi[2] ZHAO Wen-Ming[1]

[1] (*School of Life Science and Technology, Xi'an Jiaotong University, Xi'an* 710049, *China*)

[2] (*Institute of Biological Science and Technology, Hainan University, Haikou* 570228, *China*)

[3] (*Biotechnology Research Center, Zhongshan University, Guangzhou* 510275, *China*)

Abstract Soil salinity is an important issue, as most crop plants are low in salt tolerance. Salt tolerance, a complex, multifactorial, and multigenic process, has been known to be a quantitative trait. The identification of the salt stress responsive genes or salt tolerance genes is essential for the breeding programs. Most recent efforts have been focused on the products of structural genes (transport proteins, ion channels, enzymes of solute synthesis) while little attention were paid to the regulatory aspects of these proteins. Since the first aquaporin gene from plants was cloned and functionally expressed in 1993, there has been a growing interest in the molecular biology of MIPs (membrane intrinsic proteins) and their bearing on the biophysics of water flow across plant membranes. In the last decades, studies on Mangroves, a special kind of wood plants, grow in high-salt and flooding conditions have been concentrated almost exclusively on their physiological and ecological characteristics. *Kandelia candel*, one of the dominant species of mangroves along the Chinese coast, lacks salt glands or salt hairs used for removal of excess salt in other mangroves. This makes *K. candel* a perfect model to study the molecular mechanism of salt tolerance in mangrove plants. Using cDNA RDA, a cDNA-specific modification of genomic representational difference analysis, a series of salt responsive genes of *Kandelia candel* were cloned. Among these gene fragments, a 183 bp fragment (termed as SRGKC1) encoding a tonoplast intrinsic protein (TIP) in *Kandelia candel* (KCTIP1) was identified. Based on the sequence of SRGKC1, two gene specific primers were designed, and the 3′ and 5′end of the KCTIP1 gene were obtained using the SMART™ RACE cDNA Amplification Kit. RACE products were purified from low-melting agarose, and sequenced directly with GSPs as the sequencing primers. A 500-bp fragment corresponding to the 3′end of this gene was obtained using the GSP1 primer, and a 690 bp fragment corresponding to the 5′end of this gene was obtained using the GSP2 primer. Two primers that flank the putative open reading frame (ORF) were designed to obtain the cDNA containing the complete ORF by RACE PCR reaction. The full-length cDNA of KCTIP1, containing a 756 bp open reading frame (ORF), was approximately 1.1 kb; the start codon was located at the nucleotides of 99-101 and stop codon at the nucleotides of 855-857 followed by a poly (A) tail. The KCTIP1 cDNA sequence in this research was released in GenBank with accession number AF521135. Using ExPASy Proteomics tools provided by EMBL, the isoelectric point and MWt of KCTIP1 are estimated as 5.77 and 26.3 kD respectively. Transmembrane prediction analysis revealed the deduced KCTIP1 protein sequence contains six transmembrane regions at amino acid residues of 20 – 42, 57 – 79, 86 – 108, 113 – 135, 142 – 164 and 217 – 239. Two highly conserved asparagine-proline-alanine (NPA) motifs were located at 85 – 87 and 199 – 201 amino acid residues respectively. KCTIP1 is also predicted to contain the Cys residue (Cys 118) that are shown to confer Hg-sensitivity in Arabidopsis γ-TIP and δ-TIP. Similarity analysis showed that KCTIP1 shared 77% ~ 79% amino acid sequence identity with the TIPs from *Vitis berlandieri*, *Brassica oleracea* and *Arabidopsis thaliana*. Expression analyses indicated that KCTIP1 had different expression among species of Mangroves. Expressions of KCTIP1 in *Kandelia candel*, *Rhizophora apoculata* and *Ceriops tagal* were suppressed by salt, and were insensitive to salt stress in unknown species of Mangroves. Previous studied showed that salt conditions might result in large and rapid changes in extracellular water potential and serious disturbance to the cytoplasm. In order to compensate for this imbalance, the relative contribution of water channels to flow across the root could thus vary. *K. candel* is a species that is native to intertial zone of tropical and subtropical coast and is well-adapted to salt conditions. The coordinated down-regulation of aquaporins in this plant may decrease membrane water permeability and thus increase the cellular water conserva-

Received: 09-03-2002

This work was supported by Key Science and Technology Research Program of the Ninth Five-year Plan (No.85-722-27-01) and a Main Research Program of the Education Ministry of China (No.02165).

* Corresponding author. Tel: 86-898-66258650; E-mail: hnqifeng@yahoo.com.cn or wmzhao@mail.xjtu.edu.cn

tion during periods of salt stress. The results reported here are consistent with the postulated roles for tonoplast water channels in regulating the hydraulic permeability of the vacuolar membranes and in adjusting the water homeostasis of the protoplasm under various physiological conditions. The identification of KCTIP1 as one of salt-responsive genes implies that intracellular osmotic equilibration is a part of salt-tolerant mechanisms in Mangroves.

Key words Mangroves, *Kandelia candel*, tonoplast intrinsic protein, salt tolerance

Since the first aquaporin from plants was cloned and functionally expressed in 1993, there has been a growing interest in the molecular biology of MIPs (membrane intrinsic proteins) and their bearing on the biophysics of water flow across plant membranes[1]. Aquaporins are members of the membrane intrinsic protein (MIP) family, which includes tonoplast intrinsic proteins (TIPs) and plasma membrane intrinsic proteins (PIPs)[2]. Among the 23 different MIP-encoding genes identified in *Arabdopsis*, 11 are proposed to be TIPs, and 11 to be PIPs, with one (NLM1) being most closely related to soybean NOD26, a nodulin from the symbiosome membrane of soybean[3]. MIPs have six transmembrane domains and short conserved amino acid motifs, including the signature sequence SGxHxNPA, which is repeated in the second half of the protein. Structurally, the two halves of the polypeptide show observed symmetry, with the loops containing the NPA motif overlapping in the middle of the lipid bilayer to form two hemipores that together create a narrow, water-filled channel[4]. Some of these proteins transport small solutes, some transport water only, and some transport both[5].

Mangroves, a special kind of wood plants, grow in high-salt and flooding conditions. In the last decades, studies on Mangroves have been concentrated almost exclusively on their physiological and ecological characteristics[6-8]. Little attention has been drawn to the study of their gene expression patterns. There is no report on analysis of gene expression in *Kandelia candel*, a species of Mangroves.

We have isolated a 183 bp fragment from *Kandelia candel* by cDNA representational difference analysis (cDNA RDA), whose expression was down-regulated by salt. This fragment is designated SRGKC1 (Salt Repressed Gene from *Kandelia candel*), it encodes a polypeptide exhibiting 91% identity in its amino acid sequence to a gamma tonoplast intrinsic protein from *Pyrus communis*. The corresponding full-length gene is designated KCTIP1. In this paper, we report the isolation of the full-length cDNA encoding KCTIP1, and its expression patterns in response to salt stress, which is an integral part of our overall effort in elucidating the molecular mechanisms of salt tolerance in Mangroves.

1 Materials and Methods

1.1 Plant material

Viviparous seeds of *K. candel* (Rhizophoraceae) were collected from Hainan province, and planted in each pot with liquid fertilizer. The plants were irrigated every 3 days with water. After growing for 2 months, young plants were transferred from liquid fertilizer to a medium containing 3% NaCl for 12 h, and then total RNA was extracted from the root system.

1.2 Isolation of total RNA and mRNA purification

Total RNA was isolated by the CTAB method. Roots were ground in liquid nitrogen, and incubated at 65℃ for 15 min in 20 mL extraction buffer (20% CTAB, 100 mmol/L Tris-HCl (pH8.0), 20 mmol/L EDTA, 1.4 mol/L NaCl, 4% 2-mercaptoethanol). An equal volume of chloroform∶isoamyl-alcohol (24∶1, *V*/*V*) was added to the suspension, and centrifuged at 11 500 × g for 15 min. The extraction and centrifugation step was repeated once. RNA was selectively precipitated and purified by LiCl (2 mol/L) precipitation overnight at −20℃, and recovered by centrifugation at 11 500 × g for 1.5 h. The RNA pellet was washed twice in 20 mL 75% ethanol at 11 500 × g for 20 min. Then the pellet was resuspended in 200 μL diethyl pyrocarbonate (DEPC)-treated water. Poly (A)$^+$ mRNA was isolated using poly AT tract^R mRNA Isolate Kit.

1.3 3′- and 5′- RACE cloning of the 3′- and 5′-end of the KCTIP1 gene

RACE-Ready-cDNA was synthesized followed the manufacturer instructions using the SMART^{TM} RACE cDNA Amplification Kit (Clontech). A total 150 ng poly (A)$^+$ RNA was used as the template in the RACE cDNA synthesis reaction. 3′- and 5′-RACE gene specific primers (GSP) were designed based on the sequence of SRGKC1. GSP1∶ 5′-CGCCAATCGCCATCGGTTTCAT-3′, GSP2∶ 5′-AGCCCCTCCCGCCAAAACGTT-3′, Universal primer∶ 5′-CTAATACGACTCACTATAGGGC-3′. 3′-and 5′-RACE reaction were carried out using the Advantage 2 Polymerase Mix and following the manufactures instruction. Then, two primers GSP3∶5′ GCGGCCGGCGTAAAAAAAGACAGT 3′, GSP4∶ 5′ GCTCAAAACCACCAAGATAAATATACC 3′, that flank the putative open reading frame (ORF) were designed to obtain the cDNA containing the complete ORF.

1.4 Sequence analysis of the 3′- RACE and 5′-RACE products

3′- and 5′-RACE PCR products were purified by low-melting agarose, products were then sequenced directly with GSPs as sequencing primers. The acquired sequences were compared to those in Genbank using the BLAST program and analyzed with software provided by EMBL.

1.5 Northern analysis

Twenty-five μg of total RNA was electrophoresed in a denaturing formaldehyde agarose gel. Following capillary transfer overnight, the Hybond H$^+$ nylon membrane was fixed at 80℃ for 2 hours, and was

probed with a fragment carrying the KCTIP1 gene with the random primed DNA labeling KIT (Promega). The filter was washed twice in 2× SSC, 0.1% SDS at room temperature, followed by two 15 min washes in 0.2 × SSC, 0.1% SDS and 0.1 × SSC, 0.1% SDS at 65℃, and exposed to X-ray film with a high speed intensifying screen at −80℃ for 1 ~ 3 day.

2 Results

2.1 Isolation of KCTIP1 cDNA and sequence analysis

To obtain the full-length cDNA encoding KCTIP1, 3′- and 5′-RACE were performed to isolate its 3′- and 5′-end fragments. A 500 bp fragment corresponding to the 3′end of this gene was amplified using the GSP1 primer, and a 690 bp fragment corresponding to the 5′ end of this gene was amplified using the GSP2 primer. A fragment of 870 bp was then amplified using the GSP3 and GSP4 primers, which were designed based on the sequence of the 5′end and 3′end fragments.

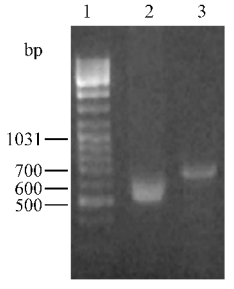

Fig.1 Identification of 5′end and 3′end RACE amplification products
1. Marker GeneRuler™ DNA ladder Mix; 2. 3′-RACE product; 3. 5′-RACE product

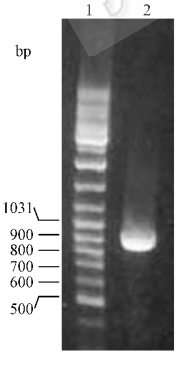

Fig.2 Identification of the full-length amplification products
1. Marker GeneRuler™ DNA ladder Mix; 2. Full-length KCTIP1

The 3′- and 5′-end fragments were ligated to give a 1099 bp cDNA fragment with a 756 bp open reading frame (ORF), encoding a protein of 252 amino acids with isoelectric point of 5.77 and MW of 26.3 kD[9]. Start codon was located at the nucleotides of 99 − 101 and stop codon at the nucleotides of 855 − 857, which was followed by a poly (A) tail. Sequence analysis indicated that this protein contains six transmembrane regions at amino acid residues of 20 − 42, 57 − 79, 86 − 108, 113 − 135, 142 − 164 and 217 − 239. Two highly conserved asparagine-proline-alanine (NPA) motifs were located at 85 − 87 and 199 − 201 amino acid residues, respectively[10] (Fig. 3). KCTIP1 are predicted to contain Cys residues (Cys 118) that are shown to confer Hg-sensitivity in Arabidopsis γ-TIP and δ-TIP[11]. The KCTIP1 cDNA sequence in this research was released in GenBank with accession number: AF521135.

The predicted amino acid sequence of KCTIP1 is 77% identical to γ-TIP from *Pyrus communis* (accession number, AB048248), *Brassica oleracea var. botrytis* (accession number, U92651) and *Arabidopsis thaliana* (accession number, NM-129238, X63552), but is most like (79% identical) a putative aquaporin TIP3 from *Vitis berlandieri x Vitis rupestris* (accession number, AF271660).

```
1    GTACCTTAGACTCTGAGCACTCTTTCCAGCTGCGGCCGGCGTAAAAAAAGACAGTAATAAGTAGCCTTTC
71   ACCGTTTGGTCTTGGTCGGCCGACGAAG ATG CCG ATC CGA CAA ATA GCC GTA GGC CAT
                                  M   P   I   R   Q   I   A   V   G   H
129  CCC CAC GAG GCA ACC CGC CCT GAC GTC TTG AAG GCA GCA CTG GCC GAG TTC
       P   H   E   A   T   R   P   D   V   L   K   A   A   L   A   E   F
180  ATC TCC ACC CTT ATT TTC GTA TTC GCC GGC GAA GGT TCC GGC ATG GCC TTC
       I   S   T   L   I   F   V   F   A   G   E   G   S   G   M   A   F
231  AAT AAA CTA ACC GAC AAC GGC TCC ACC ACA CCC GCC GGC CTT ATC GCC GCA
       N   K   L   T   D   N   G   S   T   T   P   A   G   L   I   A   A
282  TCC ATT GCC CAC GCC TTC GGT CTT TTT GTG GGC GTC TCG GTT AGC GCC AAC
       S   I   A   H   A   F   G   L   F   V   G   V   S   V   S   A   N
333  ATC TCA GGT GGC CAC GTT AAC CCC GCC GTC ACT TTC GGT GCC TTT ATC GGT
       I   S   G   G   H   V   N   P   A   V   T   F   G   A   F   I   G
384  GGA AAC ATC ACT CTT CTC CGG GGT ATC CTT TAC TGG ATT GGT CAG CTC CTC
       G   N   I   T   L   L   R   G   I   L   Y   W   I   G   Q   L   L
435  GGC TCC ACA GTC GCC TGC CTG CTT CTC AAG TTC TCC ACC GGC GGC CTG ACA
       G   S   T   V   A   C   L   L   L   K   F   S   T   G   G   L   T
486  ACC TCG GCC TTT TCG CTT TCC TCT GGA GTT AGC GTA TGG AAC GCG TTT GTT
       T   S   A   F   S   L   S   S   G   V   S   V   W   N   A   F   V
537  TTC GAG ATA GTC ATG ACC TTC GGC CTC GTG TAC ACA GTC TAC GCG ACG GCT
       F   E   I   V   M   T   F   G   L   V   Y   T   V   Y   A   T   A
588  ATT GAT CCC AAG AAG GGA AAT TTG GGC ACA ATC GCG CCA TCG CCA TCG GTT
       I   D   P   K   K   G   N   L   G   T   I   A   P   S   P   S   V
639  TCA TTG TGG GTG CCT AGT GTT TGG ACG GGA GGG GCT TTC GAC GGA GCC TCA
       S   L   W   V   P   S   V   W   T   G   G   A   F   D   G   A   S
690  ATG AAC CCC GCC GTA TCA TTT GGG CCT GCT TTG GTG AGT TGG ACA TGG GAG
       M   N   P   A   V   S   F   G   P   A   L   V   S   W   T   W   E
741  AAC CAC TGG GTG TAC TGG GCC GGT CCT CTG ATC GGC AGT GCT ATT GCT GCT
       N   H   W   V   Y   W   A   G   P   L   I   G   S   A   I   A   A
792  CTC ATT TAT GAT TCC TTC TTC ATC GGC TAT GGC ACC CAC GAG CAG CTC CCC
       L   I   Y   D   S   F   F   I   G   Y   G   T   H   E   Q   L   P
843  ACC GCT GAC TAC TAAGCATATGGACAGGTTGACATCTGCATAATGCCTCCGGCTTTCTCGCTCTTT
       T   A   D   Y
909  TCTTGGTATATTTATCTTGGTGGTTTTGAGCTTTGTTATTTTAGCATCTGTTCAATTTGCCGTAGTTGGG
979  GTTTCCAGTTTCAGTTGTGTTGCTTTTCTTTTTCTTTTCTTTGTTTTTTCATTCCTTCTCGTTTAATTTC
1049 CAATTTCAAATTCATGTCTTTTGATTAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig.3 The nucleotide and its predicted amino acid sequence of KCTIP1 cDNA

The signal peptide is underlined, transmembrane regions are shown in shadow, and highly conserved asparagine-proline-alanine (NPA) motif is boxed.

2.2 Expression analysis of KCTIP1

The expression patterns of KCTIP1 in different species of Mangroves were investigated by Northern blot analyses. SRGKC1 was used as the hybridization probe on blots containing total RNAs from salt-treated and control samples.

As shown in Fig.5A − D, KCTIP1 had different expression

```
Kandelia candel                  MPIRQIAVGHPHEATRPDVLKAALAEFISTLIFVFAGEGS  40
Vitis berlandieri x Vitis rupestris  MPIYRIAIGTPGEASHPDALKAALAEFFSMLIFVFAGEGS
Pyrus communis                   MPIRNIAVGRPEETYHPDALRAALAEFISTLIFVFAGEGS
Brassica oleracea var. botrytis  MPIRNIAVGRPDEATRPDALKAALAEFISTMIFVFAGSGS
Arabidopsis thaliana             MPIRNIAIGRPDEATRPDALKAALAEFISTLIFVVAGSGS

Kandelia candel                  GMAFNKLTDNGSTTPAGLIAASIAHAFGLFVGVSVSANIS  80
Vitis berlandieri x Vitis rupestris  GMAFNKLTDSGSSTPAGLVAAALAHGFALFVAVSVGANIS
Pyrus communis                   GMAFAKLTDDAANTPAGLVAAALAHAFGLFVAVSISANIS
Brassica oleracea var. botrytis  GMAFNKLTENGATTPAGLVAASLAHAFGLFVAVSVGANIS
Arabidopsis thaliana             GMAFNKLTENGATTPSGLVAAAVAHAFGLFVAVSVGANIS

Kandelia candel                  GGHVNPAVTFGAFIGGNITLLRGILYWIGQLLGSTVACLL  120
Vitis berlandieri x Vitis rupestris  GGHVNPAVTFGAFLGGHITLLRGILYWIAQLLGSVVACLL
Pyrus communis                   GGHVNPAVTFGAFIGGNISLLRGILYWIAQLLGATVACGLL
Brassica oleracea var. botrytis  GGHVNPAVTFGAFVGGNITLLRGILYWIAQLLGSVVACLL
Arabidopsis thaliana             GGHVNPAVTFGAFIGGNITLLRGILYWIAQLLGSVVACLI

Kandelia candel                  LKFSTGGLTTSAFSLSSGVSVWNAFVFEIVMTFGLVYTVY  160
Vitis berlandieri x Vitis rupestris  LKFSTGGLETSAFSLSSGVSVWNALVFEIVMTFGLVYTVY
Pyrus communis                   LKFVTNGQTTAAFSLSSGVGVWNAFVFEIVMTFGLVYTVY
Brassica oleracea var. botrytis  LKFTTGGLVVPAFGLSAGVGVSNALVFEIVMTFGLVYTVY
Arabidopsis thaliana             LKFATGGLAVPAFGLSAGVGVLNAFVFEIVMTFGLVYTVY

Kandelia candel                  ATAIDPKKGNLGTIAPSPSVSLWVPSVWTGGAFDGASMNP  200
Vitis berlandieri x Vitis rupestris  ATAVDPKKGNLGIIAPIAIGFIVGANILAGGAFDGASMNP
Pyrus communis                   ATAIDPKNGSVGTIAPIAIGFIVGANILAGGAFDGASMNP
Brassica oleracea var. botrytis  ATAVDPKNGSLGTIAPIAIGFIVGANILAGGAFSGASMNP
Arabidopsis thaliana             ATAIDPKNGSLGTIAPIAIGFIVGANILAGGAFSGASMNP

Kandelia candel                  AVSFGPALVSWTWENHWVYWAGPLIGSAIAALIYDSFFIG  240
Vitis berlandieri x Vitis rupestris  AVSFGPAVVSWSWANHWVYWAGPLIGAAIAAIIYDHIFID
Pyrus communis                   AVSFGPALVSWSWENHWVYWAGPLIGAGLAGLIYEFVFIG
Brassica oleracea var. botrytis  AVAFGPAVVSWSWNNHWVYWAGPLVGGGLAGLIYEVFFIN
Arabidopsis thaliana             AVAFGPAVVSWTWTNHWVYWAGPLVGGGIAGLIYEVFFIN

Kandelia candel                  YGTHEQLPTADY                              252
Vitis berlandieri x Vitis rupestris  -NTHEQLPTTDY
Pyrus communis                   NSGHEQLPSTDY
Brassica oleracea var. botrytis  T-THEQLPTADY
Arabidopsis thaliana             T-THEQLPTTDY
```

Fig.4 Comparison of KCTIP1homologues from different species, *Vitis berlandieri x Vitis rupestris*, *pyrus communis*, *brassica oleracea var botrytis* and *Arabidopsis thaliana*

among species of Mangroves. Expressions of KCTIP1 in *Kandelia candel*, *Rhizophora apoculata* and *Ceriops tagal* were suppressed by salt, and displayed insensitive to salt stress in unknown specie of Mangroves. The hybridization membrane for *K. candel* in Fig. 5A was used repeatedly and signal was obscure, thus, time-course analysis of KCTIP1 expression in *K. candel* following 3% NaCl treatment was performed. As shown in Fig. 5E, the expression of KCTIP1 decreased gradually with increased NaCl treatment time.

3 Discussion

This paper is the first report of TIP gene family in Mangroves. The deduced amino acid sequence of KCTIP1 contains all the features typical of aquaporins, including residues that may confer Hg sensitivity to the putative TIPs, six transmenbrane regions and two NPA motifs that might create a narrow, water-filled channel.

Studies of the aquaporin gene family are most complete for *A. thaliana*[3] and *M. Crystallinum*[4]. The deduced amino acid se-

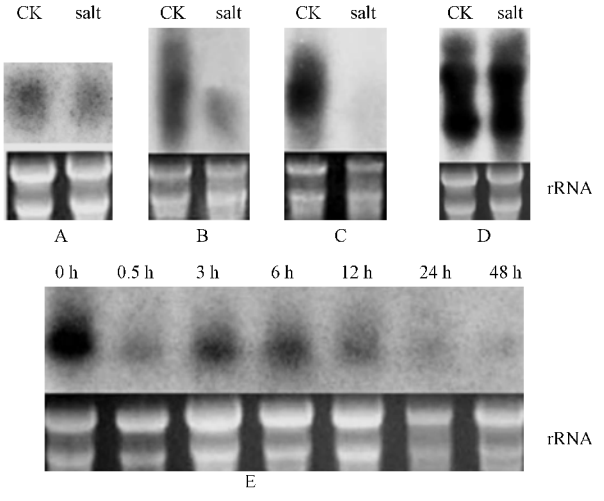

Fig.5 Northern blot analysis of SRGKC1

A ~ D. Northern blot analysis of SRGKC1 in *Kandelia candel* (A), *Rhizophora apoculata* (B), *Ceriops tagal* (C), unknown species (D), respectively. CK: Control plants (not treated with 3% NaCl); salt: Salt treated plants (3% NaCl treatment for 12 h). (E) Time-course analyses of SRGKC1 expression after 3% NaCl treatment. Total RNAs was isolated from plants after treatment with 3% NaCl for 0, 0.5, 3, 6, 12, 24 and 48 h, respectively and hybridized with the radioactively labeled SRGKC1. Approximately 20 μg of total RNA from each sample was used for Northern blot analysis. Ethidium bromide stained rRNAs were used as loading control.

quence of KCTIP1 is 77% identical to that of a γ-TIP from *A. thaliana* and 72% identical to *M. crystallinum*. The discovery of water-channel proteins in membranes of plant cells reveals potential new mechanisms that may be used by plants to control water transport and osmotic adjustment[12]. Aquaporins have two postulated functions: as TIPs in intracellular osmotic equilibration and as PIPs in the regulation of the transcellular water transport[13].

There are various isoforms of TIPs: alpha (seed), gamma (root), and Wsi (water-stress induced). These proteins may allow diffusion of water, amino acids and/or peptides from the tonoplast interior to the cytoplasm[14-18]. Salt conditions may result in large and rapid changes in extracellular water potential and serious disturbance to the cytoplasm.

In order to compensate for this imbalance, the relative contribution of water channels to flow across the root could thus vary[4]. The expression of KCTIP1 was repressed by salt. *K. candel* is a species that is native to intertial zone of tropical and subtropical coast and is well-adapted to salt conditions. The coordinated down-regulation of aquaporins in this plant may decrease membrane water permeability and thus increase the cellular water conservation during periods of salt stress. These results are consistent with the postulated roles for tonoplast water channels in regulating the hydraulic permeability of the vacuolar membranes and in adjusting the water homeostasis of the protoplasm under various physiological conditions[13, 19]. KCTIP1 is one of salt-responsive genes isolated from *K. candel*, which implies that the role for TIP in intracellular osmotic equilibration is a part of salt-tolerant mechanisms in Mangroves. The isolation of cDNA encoding KCTIP1 and its expression laid the foundation for salt tolerance research for Mangroves.

REFERENCES(参考文献)

[1] Maurel C, Reizer J, Schroeder J I, Chrispeels M J. The vacuolar membrane protein γ-TIP creates water specific channels in *Xenopus oocytes*. *The EMBO Journal*, 1993, **12**: 2241 – 2247

[2] Anthony Yeo. Molecular biology of salt tolerance in the context of whole-plant physiology. *Journal of Experimental Botany*, 1998, **49** (323): 915 – 929

[3] Weig A, Deswarte C, Chrispeels M J. The major intrinsic protein family of *Arabidopsis* has 23 membranes that form three distinct groups with functional aquaporins in each group. *Plant Physiology*, 1997, **114**: 1347 – 1357

[4] Tyerman S D, Bohnert H J, Maurel C, Steudle E, Smith J A C. Plant aquaporins: their molecular biology, biophysics and significance for plant water relations. *Journal of Experimental Botany*, 1999, **50**: 1055 – 1071

[5] Park J H, Saier MH Jr. Phylogenetic characterization of the MIP family of transmembrane channel proteins. *J Memb Biol*, 1996, **153**: 171 – 180

[6] Mei Sun, Wong K C, Joe S Y Lee. Reproductive biology and population genetic structure of Kandelia candel (Rhizophoraceae), a viviparous mangrove species. *American Journal of Botany*, 1998, **85**(11): 1631 – 1637

[7] Elizabeth J, Farnswrth, Jili M Farrant. Reductions in abscisic acid are linked with viviparous reproduction in mangroves. *American Journal of Botany*, 1998, **85**(6): 760 – 769

[8] LIN P (林鹏). The physiological characteristics of salt tolerance for kandelia candel seedlings. In: Mangroves research papers (III) (**红树林研究论文集**), Xiamen University Press, 1999, pp.150 – 154 (in Chinese)

[9] Wilkins M R Gasteiger E, Bairoch A, Sanchez J-C, Williams K L, Appel R D, Hochstrasser D F. Protein Identification and Analysis Tools in the ExPASy Server. in: 2 – D Proteome Analysis Protocols, 1998, Editor A.J. Link. Humana Press, New Jersey

[10] Schultz J, Milpetz F, Bork P, Ponting C P. SMART, a simple modular architecture research tool: identification of signaling domains. *Proc Natl Acad Sci U S A*, 1998, **95**(11): 5857 – 5864

[11] Daniels M J, Chaumont F, Mirkov T E, Chrispeels M J. Characterization of a new vacuolar membrane aquaporin sensitive to mercury at a unique site. *The Plant Cell*, 1996, **8**: 587 – 599

[12] Maurel C. Aquaporins and water permeability of plant membranes. *Annu Rev Plant Physiol Plant Mol Biol*, 1997, **48**: 399 – 429

[13] Fran?ois Barrieu, Fran?ois Chaumont, Maarten J Chrispeels. High expression of the tonoplast aquaporin ZmTIP1 in epidermal and conducting tissues of maize. *Plant Physiol*, 1998, **117**: 1153 – 1163

[14] Johnson K D, Herman E M, Chrispeels M J. An abundant, highly conserved tonoplast protein in seeds. *Plant Physiol*, 1989, **91**: 1006

[15] Jocelyn A Ozga, Rika van Huizen, Dennis M Reinecke. Hormone and Seed-Specific regulation of pea fruit growth. *Plant Physiol*, 2002, **128**:1379 – 1389

[16] Melroy D L, Herman E M. TIP an integral membrane protein of the protein-storage vacuoles of the soybean cotyledon undergoes developmentally regulated membrane accumulation and removal. *Planta*, 1991, **184**:113 – 122

[17] Ludevid D, Höfte H, Himelblau E, Chrispeels M J. The expression pattern of the tonoplast intrinsic protein -TIP in *Arabidopsis thaliana* is correlated with cell enlargement. *Plant Physiol*, 1992, **100**:1633 – 1639

[18] Guang-Yuh Jauha, Thomas E Phillipsb, John C Rogersa. Tonoplast intrinsic protein isoforms as markers for vacuolar functions. *Plant Cell*, 1999, **11**:1867 – 1882

[19] Maurel C, Chrispeels M J, Lurin M, Tacnet F, Geelen D, Ripoche P, Guern J. Function and regulation of plant seed aquaporins. *J Exp Bot*, 1997, **48**:421 – 430